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The Effect of Agitation on the Rate of Acid Formation by *Lactobacillus delbrueckii**

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Summary. The effect of agitation on the rate of acid formation by the microaerophilic organism *Lactobacillus delbrueckii* was studied. Fermentations were conducted at constant temperature and pH, and with the continuous addition of a nutrilite source. The range of agitator speeds was from 85 to 720 rev/min.

The acid formation rate was found to increase with increasing agitator speeds and decrease with decreasing speeds if corn-steep liquor was the nutrilite source. No effect of agitation was observed with yeast extract as the nutrilite source.

The data from corn-steep liquor media were found to be consistent with a mass transfer mechanism, but did not warrant a conclusion as to the specific nature of the effect of agitation. It was concluded, however, that the effect of agitation on the acid formation rate was at most very small.

Introduction

This work was undertaken to study quantitatively the effect of mechanical agitation on the rate of lactic acid formation by the homofermentative organism *Lactobacillus delbrueckii*.

Numerous references on the effects of agitation (and aeration) on the rates and yields of aerobic fermentations may be found in the literature. The comprehensive review by Finn[1] indicates the extensive interest in this subject. There is very little

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published work, however, on the effects of agitation on anaerobic and microaerophilic organisms.

Rogers and Whittier,[2] and Rahn *et al.*[3] have reported on the effects of stirring on the growth of the facultative anaerobe *Streptococcus lactis*. The former reported that mechanical agitation, bubbling air, and bubbling nitrogen produced, in that order, higher cell populations than were obtained in unstirred cultures. The latter reported that agitation with air or oxygen decreased the fermentation rate, while agitation with nitrogen increased the rate, compared with unstirred cultures. Cutter[4] compared fermentations at different rates of mechanical agitation and concluded that the only apparent effect of agitation on the anaerobic fermentation of glucose by yeast was to decrease the length of the lag phase.

In view of the scant information on non-aerobic fermentations, this work was initiated to study the effect of agitation on the rate of fermentation by a microaerophilic organism. *L. delbrueckii* was selected for this investigation for several reasons. First, this organism is considered to be microaerophilic,[5] that is, some oxygen is required for growth, but the organism prefers a lower oxygen tension than that when exposed to the air;[6, 7] hence, it was not necessary to bubble air through the media. Certain results to be cited later in this paper indicate that *L. delbrueckii* NRRL B–445 is indifferent to the oxygen concentration. Secondly, considerable work has been done with this organism, and a convenient and accurate method has been developed for determining the fermentation rate.[8] Thirdly, it has been observed that under properly controlled conditions, the total rate of acid formation in the fermentor could be maintained at a constant value for a considerable period of time.[9, 10]

In order to obtain a constant rate, the temperature and pH were closely controlled, the agitator speed was held constant, and a source of nutrient materials was added to the fermenting medium essentially continuously throughout the course of a fermentation.

This period of constant fermentation rate is particularly valuable, since it allows the observation of the effect of a change of a single variable on the rate of fermentation during the course of a single fermentation.

Equipment and Methods

The equipment employed was the same as that described by Gillies and Kempe,[11] but modified to allow vigorous agitation and variation of the agitator speed. Agitation was accomplished with a vaned-disc impeller driven through a belt-and-pulley

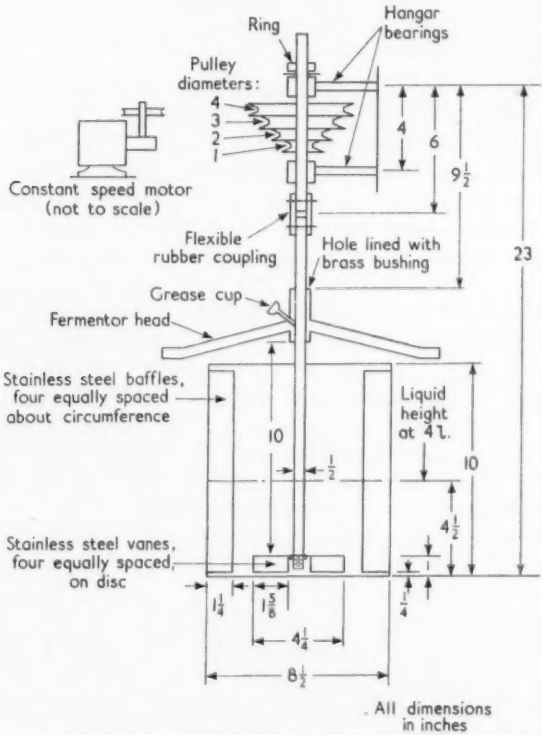

Fig. 1. Details of fermentor interior and agitation system

system by a 1/20 h.p. motor. The details of the fermentor interior and agitation system are shown in Fig. 1. The agitation system was such that the agitator speed could be varied from 85 to 720 rev/min. Four stainless steel baffles equally spaced about the periphery of the vessel provided sufficient baffling so that

vortexing did not occur, even at the highest agitator speeds employed.

The temperature of the medium was controlled within $\pm 0{\cdot}2$°C by two electric heaters in a water bath surrounding the fermentor. One heater was actuated by a bi-metallic thermoregulator in the bath and the other by a mercury thermoregulator in the fermentor. The pH was controlled within $\pm 0{\cdot}03$ units by a Beckman Model RX pH meter and a Bristol Pyromaster potentiometer.

Acid formed by the organisms was neutralized with 2 N sodium hydroxide, pumped by a Sigmamotor pump* whenever the pH fell below the control limit. The neutralizing solution was stored in a 1 l. graduated cylinder and the volume of solution remaining was recorded on a revolving kymograph drum. The resulting curve of alkali volume *versus* time was graphically differentiated to determine the instantaneous rate of addition of alkali solution. The total rate of acid formation in the fermentor was directly proportional to the measured rate of alkali addition. Instantaneous acid formation rates were determined with a precision of ± 1 mequiv/h. It was found that 95 per cent of the titrable acid was lactic (as determined by the method of Friedmann and Graeser[12]). It should be noted that acid formation rates calculated from the rates of alkali addition were total rates, not rates per unit volume. It was the total rate which remained constant in control runs.

The basal medium was the same as that described previously,[11] except that 150 or 180 g of glucose were used. Either corn-steep liquor or yeast extract was used as a source of required and stimulatory nutrilites. The corn-steep liquor used in this work was treated prior to sterilization by the method described by Liggett and Koffler.[13] A quantity of the nutrilite source was added to the basal medium and additional nutrilites were added semi-continuously throughout the course of the fermentation by the Sigmamotor pump.[10] Since the pump operated approximately 15 sec of each 2 min when a fermentation was progressing actively, the nutrilite addition was essentially continuous. It was this continuous addition of nutrilites which allowed the establishment of a constant rate of acid formation.

* Sigmamotor Inc., Middleport, New York.

L. delbrueckii NRRL B–445 was used throughout the investigation. This organism is classified as microaerophilic.[5]

The experimental procedure followed that described earlier,[10, 11] except that the agitator speed was changed periodically during a single fermentation by varying the pulley diameter ratio. The temperature and pH were controlled at the same level throughout a run. The procedure of changing the agitator speed took less than 5 min, so no significant changes in the medium occurred during this operation. A period of 4 to 6 h at each agitator speed was allowed so that a constant rate would be established. Only three or four different agitator speeds could be used during a single run, since the duration of the constant rate period was usually about 20 h.

Results

During the course of this work it was repeatedly found, in accordance with the observations of Gillies,[9] that a constant rate of acid formation was established by *L. delbrueckii* if the temperature, pH, and agitator speed were held constant and nutrilites were added continuously. This constant rate was observed both in corn-steep liquor and yeast extract media.

In corn-steep liquor media the acid formation rate changed measurably with changes in agitator speed; in yeast extract media this effect was not observed. It should be noted that all rates under discussion are total rates, not rates per unit volume.

Fig. 2 shows the results of a typical run in a corn-steep liquor medium. The acid formation rate, in mequiv/h, is plotted as a function of the time after inoculation; periods at various agitator speeds are indicated by horizontal arrows. Fig. 2 shows that when the agitator speed was increased from 85 to 720 rev/min, the acid formation rate increased from 49·5 to 55 mequiv/h. A decrease in agitator speed from 720 to 275 rev/min was followed by a decrease in rate from 55 to 53 mequiv/h. When the speed was returned to the original value of 85 rev/min the rate returned to its original value of 49·5 mequiv/h. These results illustrate that each change in agitator speed in the range investigated resulted in a rate change. Furthermore, the effect of agitation was reversible, i.e. upon returning the speed to a previous value after

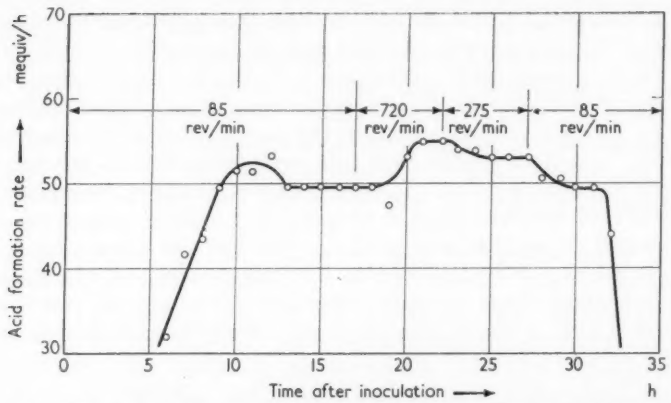

Fig. 2. Effect of agitator speed on the rate of acid formation by *L. delbrueckii*, in a glucose–corn-steep liquor medium at 43·4°C and pH 5·40

a change, the corresponding previous rate was established. It is also interesting to note that when the agitator speed was changed a new constant rate was not established for a period of 2 to 4 h, although a change in rate was noticed almost immediately.

Several runs were made with corn-steep liquor media and the aforementioned results were consistently observed and were reproducible. The results from several runs are summarized in Table I.

Table I. The effect of agitator speed on the rate of acid formation by *L. delbrueckii* in glucose–corn-steep liquor media at 43·4°C

Run	pH	Agitator speed, rev/min	Rate of acid formation, mequiv/h
24	5·45	85	49
		550	56
25	5·40	85	49·5
		720	55
		275	53
26	5·53	85	56·5
		275	60
		550	67
27	5·40	85	53
		275	58·5
		430	65·5

In media employing yeast extract as the nutrilite source there was no consistent effect of agitation. Fig. 3 shows the results of one run wherein yeast extract was used. Examination of Fig. 3 shows no effect attributable to agitation. When the agitator speed was changed from 85 to 170 rev/min the rate had not yet become constant, but did establish a constant value at 170 rev/min. An increase in agitator speed to 320 rev/min caused no change in rate for about 5 h, at which time slight increase occurred. After the rate had increased at 320 rev/min a change to 85 rev/min caused no further rate change. This plot is representative of the

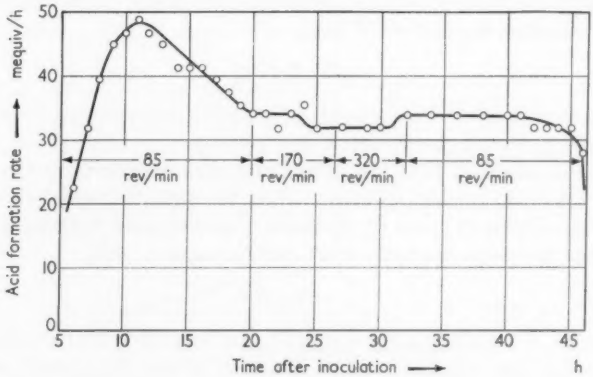

Fig. 3. Effect of agitator speed on the rate of acid formation by *L. delbrueckii*, in a glucose–yeast extract medium at 43·5°C and pH 5·60

results obtained in yeast extract media, in that no consistent or reproducible effect of agitation was observed. Furthermore, the changes which sometimes did occur were small in magnitude. It was concluded that agitation had no measurable effect on the fermentation rate in yeast extract media.

A possible mechanism for the observed effect of agitation in corn-steep liquor media is a change in the liquid phase mass transfer coefficient (see discussion). In accordance with this mechanism, the acid formation rate should be proportional to a liquid film mass transfer coefficient, the total organism–liquid interfacial area, and a driving force given by the difference

between the bulk and interfacial concentrations of a rate-limiting material, according to the equation,

$$r = k_L A(C - C_i) \tag{1}$$

where r is the acid formation rate, k_L is a liquid film mass transfer coefficient, A is the total organism–liquid interfacial area, and C and C_i are the bulk and interfacial concentrations of a rate-limiting material, respectively.

Empirical correlations for solid–liquid mass transfer coefficients in agitated vessels indicate that the mass transfer coefficient should vary exponentially with the agitator speed if the vessel and impeller dimensions and physical properties of the fluid and solid remain constant,[14, 15] thus,

$$k_L = k_1(R)^a \tag{2}$$

where k_1 is a constant for a given set of experimental conditions, R is the agitator speed, and a is a constant.

If it is assumed that the interfacial area and driving force (or their product) remain constant when the agitator speed changes, then equations (1) and (2) indicate that the acid formation rate should vary exponentially with agitator speed, thus,

$$r = k_2(R)^a \tag{3}$$

where k_2 is a constant for a given set of experimental conditions. Note that the constant k_2 is a function of the dimensions of the vessel and impeller, the physical properties of the fluid and solid, the interfacial area, the medium composition, and the temperature and pH.

According to the postulated mechanism and aforementioned assumptions, a plot of the acid formation rate *versus* the agitator speed should be linear on logarithmic co-ordinates. When the data of Table I were plotted on logarithmic co-ordinates it was found that the points for each run scattered about a different straight line. However, each line had approximately the same slope (equal to the exponent, a), indicating that the constant k_2 was different for each run.

Because of the few points available for any single run, the data were re-plotted so that a single line should fit the data for all the runs. Since for any one run k_2 should be constant, if the ratio of

the acid formation rate at one agitator speed to that at another is plotted against the corresponding agitator speed ratio on logarithmic co-ordinates, a straight line should result with slope a, as shown by the equation

$$\frac{r_1}{r_2} = \left(\frac{R_1}{R_2}\right)^a \tag{4}$$

where r_1 and r_2 are the acid formation rates at agitator speeds R_1 and R_2, respectively. Equation (4) should apply to all runs, since the coefficient k_2 does not appear.

Fig. 4 is a plot of the ratio of acid formation rates *versus* the ratio of the corresponding agitator speeds on logarithmic co-ordinates. Fig. 4 shows that there is considerable scatter in the

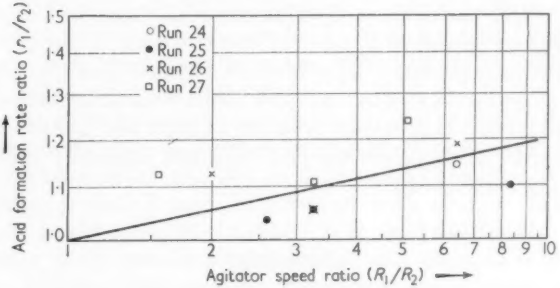

Fig. 4. Ratio of acid formation rates by *L. delbrueckii* as a function of the corresponding ratio of agitator speeds in glucose–corn-steep liquor media.

data, but that the points follow the trend of the 'least-square' line shown on the plot. A t test at the 95 per cent confidence level showed that the 'least-square' line could pass through the point (one, one) as would be expected from the results shown in Fig. 2. The slope of the line in Fig. 4 was found by 'least-squares' to be 0·08, hence equation (4) becomes

$$\frac{r_1}{r_2} = \left(\frac{R_1}{R_2}\right)^{0.08}$$

Although *L. delbrueckii* is classified as microaerophilic, certain observations during this work indicate that the particular organism used was not sensitive to the oxygen concentration. For

example, a uniform number and size of colonies was observed throughout rolled Prickett tubes containing a freshly sterilized medium composed of 0·5 per cent glucose, 2 per cent yeast extract, and 1 per cent agar. Since the medium should have been nearly devoid of oxygen after sterilization, oxygen would be supplied only by diffusion through the agar. There would then be an oxygen concentration gradient from the top to the bottom of the tubes. If the oxygen concentration had an appreciable effect on the growth of this organism, a gradient in the number and/or size of colonies should have been observed. The absence of such a result therefore indicates little or no effect of oxygen concentration.

The absence of a noticeable effect of oxygen was also observed in stab cultures in the same medium as used for the rolled tubes, and in Petri plates as well. The medium used in the plating work was composed of 2 per cent yeast extract, 2 per cent agar, and 1 per cent glucose. Furthermore, the fact that the organism grew well in the fermentor in liquid media containing either corn-steep liquor or yeast extract without an inert gas blanket demonstrates that atmospheric oxygen does not markedly inhibit its growth in these media.

Discussion

It seems worth while to consider the significance of the constant rate of acid formation that developed in fermentations continuously supplied with nutrilites. In addition to this result, it was observed that if the same amount of nutrilite source was added initially to the basal medium and none was added continuously, the rate increased to a maximum and then steadily decreased throughout the fermentation.[10] A comparison suggests that some component or components of the nutrilite source limited the fermentation rate under the experimental conditions. That is, the rate-limiting material was disappearing from the medium as the fermentation progressed, either by being consumed by the organism or by some reaction in the medium. When the nutrilite was added at a rate equal to its rate of disappearance, its concentration, and hence the rate of acid formation, remained constant. Thus, it appears possible that liquid phase mass

transfer of this material could be a rate-limiting step in the fermentation process.

In the presentation of the results, an analysis based on a postulated liquid phase mass transfer mechanism was presented in some detail. It was shown that the data obtained in corn-steep liquor media follow a trend consistent with that analysis. This certainly does not prove that the postulated mechanism is correct. In fact, several other mechanisms for the observed phenomenon can be considered.

The effects of agitation should be on the physical condition of the organism or medium, with perhaps a concomitant chemical change. Possible mechanisms include the following: (1) a breaking of chains of organisms into single cells or smaller chains, hence producing more interfacial area; (2) a change in the physical nature of the cell wall due to abrasion; (3) the reduction of gross composition gradients in the medium; (4) an increase in the rate of liquid phase mass transfer to the cells; and (5) an increase in the gas–liquid mass transfer rate.

The first three mechanisms suggested above are not consistent with the observation that agitation had no effect in yeast extract media. If the effect was due to a physical change in the cells or medium it should have occurred in both media. A similar argument would apply to the fourth suggested mechanism, unless the nature of the rate-limiting material was different in the two media. In view of the known differences in composition of yeast extract and corn-steep liquor,[13, 16] it is reasonable to suspect that the rate-limiting material was not the same in these two nutrient source materials.

It should be recognized that objections to a liquid phase mass transfer mechanism have been proposed. Finn has argued that at most only a very small diffusional resistance should exist around single cells of micro-organisms, due to their small size; and, further, that it is unlikely that mechanical agitation could develop a sufficient relative velocity between the cells and the liquid to markedly change that resistance.[1] While his discussion applied specifically to oxygen uptake, the same argument could be extended to a non-aerobic fermentation. There is insufficient experimental evidence currently available, however, to apply this argument critically to the present fermentation.

The fifth mechanism suggested above also remains a possibility. The effect of agitation could have been to change the rate of desorption of dissolved gases (especially carbon dioxide), or of absorption of gases from the atmosphere above the liquid in the fermentor (in particular oxygen). It is quite unlikely, however, that the desorption of carbon dioxide could be a rate-limiting factor, for again, the effect of agitation should have been the same in both media.

The medium was not blanketed with an inert gas as has been the practice of some investigators.[8] Thus, there exists the possibility that oxygen was being dissolved from the air in the fermentor, and that the rate of dissolution increased with increasing agitator speed. However, this mechanism could account for the observations only if the oxygen concentration in the medium was less than the saturation value and: (1) oxygen directly affected the rate of acid formation by the organism, or (2) oxygen affected the medium composition such that the rate of acid formation would be altered. Again, since agitation did not have the same effect in the two media used, the first of these two possibilities is unlikely. The second remains a possibility, however, if oxygen reacted differently with the two nutrilite sources.

The medium should have been saturated or nearly saturated with oxygen a few hours after inoculation, due to dissolution at the air–liquid interface, unless the organism or the medium exerted an appreciable oxygen demand. Since the organism is classified as microaerophilic and certain results of this work suggest that it is indifferent to oxygen, it should not exert any appreciable oxygen demand. The possibility still remains that the medium might react with dissolved oxygen and thereby exert an 'oxygen demand'. Whether or not the composition of either medium was influenced by oxygen absorption and whether that would affect the rate of acid formation is problematical.

Longsworth and MacInnes[17] have reported that a relatively large increase in the oxygen tension decreased slightly the rate of acid formation by *L. acidophilus*. Such a reduction is consistent with the microaerophilic nature of *L. acidophilus*.[5] That is, a microaerophilic organism should prefer a reduced oxygen tension.[6, 7] Since it was observed that the rate of fermentation by

L. delbrueckii increased with increasing agitator speed, the oxygen absorption mechanism is not consistent with the results of Longsworth and MacInnes.

In view of the foregoing discussion, it is apparent that there is not sufficient information to specifically determine the mechanism of the effect of agitation on this fermentation. However, the results obtained in corn-steep liquor media are consistent with a liquid phase mass transfer mechanism. In any event, it can be stated that the effect is at most small and for most purposes probably negligible.

Conclusions

A consistent and reproducible, but small, effect of agitator speed on the rate of acid formation by *L. delbrueckii* was observed in corn-steep liquor media, but not in yeast extract media. The data are consistent with a liquid phase mass transfer mechanism, but do not warrant a conclusion concerning the mechanism.

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References

[1] Finn, R. K. *Bact. Rev.*, 18 (1954), 254
[2] Rogers, L. A. and Whittier, E. O. *J. Bact.*, 16 (1928), 211
[3] Rahn, O., Hegarty, C. P. and Deuel, R. E. *J. Bact.*, 35 (1938), 547
[4] Cutter, L. A. Chemical Engineer Degree Thesis, Columbia University, New York, N.Y. (1951)
[5] Breed, R. S., Murray, E. G. D. and Hitchens, A. P. Bergey's *Manual of Determinative Bacteriology*, 6th ed. (1948). Baltimore, Md.; Williams and Wilkins Co.
[6] Anderson, C. G. *Introduction to Bacteriological Chemistry*, 2nd ed., p. 199 (1946). Baltimore, Md.; Williams and Wilkins Co.
[7] Porter, J. R. *Bacterial Chemistry and Physiology*, p. 631 (1946). New York; Wiley and Sons
[8] Finn, R. K., Halvorson, H. O. and Piret, E. L. *Industr. Engng. Chem. (Industr.)*, 42 (1950), 1857
[9] Gillies, R. A. Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan (1955)
[10] Kempe, L. L., Gillies, R. A. and West, R. E. *Appl. Microbiol.*, 4 (1956), 175

[11] Gillies, R. A. and Kempe, L. L. *J. Agr. Food Chem.*, 5 (1957), 706
[12] Friedemann, T. E. and Graeser, J. B. *J. biol. Chem.*, 100 (1933), 291
[13] Liggett, R. W. and Koffler, H. *Bact. Rev.*, 12 (1948), 297
[14] Hixson, A. W. and Baum, S. J. *Industr. Engng. Chem.* (*Industr.*), 32 (1941) 478
[15] Johnson, A. I. and Huang, C. J. *A.I.Ch.E. Journal*, 2 (1956), 412
[16] Stokes, J. L., Gunness, M. and Foster, J. W. *J. Bact.*, 47 (1944), 293
[17] Longsworth, L. G. and MacInnes, D. A. *J. Bact.*, 31 (1936), 287

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The Course of Bacterial Population Growth in Batch and Continuous Culture According to the Theory of Monod*

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Summary. The equations of Monod that describe the course of bacterial population growth and substrate concentration during continuous culturing are usually analysed with reference to steady-state conditions. These equations are analysed to give information on the course of bacterial population growth from inoculation to the achievement of a steady-state condition. For common values of certain parameters occurring in Monod's development, growth is well approximated by an exponential law (logarithmic phase) which depends on: (a) the maximum specific growth-rate constant for the organism and medium concerned, and (b) the constant dilution rate.

Introduction

The development of continuous culturing techniques has been one of the reasons for a revival of interest in general patterns of microbial growth.[6] Naturally, in the consideration of continuous flow systems, the main interest has been in steady-state conditions. Thus, one usually finds, in the customary deterministic approach to bacterial constant-growth apparatus, that simultaneous differential equations describing bacterial and substrate concentration at all points in time are quickly manipulated to yield information concerning steady-state values.[3, 8] The purpose of this note is to examine these differential equations for information on the course of bacterial population growth from inoculation to the achievement of a steady-state level.

* Presented at the 134th National Meeting of the American Chemical Society, Chicago, September 1958.

The Differential Equations for Bacterial and Substrate Concentration in a One-Vessel Continuous Fermentor

The fundamental differential equations for conditions in the culture vessel are:

$$dx/dt = x[\mu(s) - D] \tag{1}$$

$$ds/dt = D(s_0 - s) - F(x, s) \tag{2}$$

where t = time, x = concentration of organisms by mass, s = concentration of limiting substrate, s_0 = concentration of limiting substrate in the ingoing medium—assumed constant, D = dilution rate—assumed constant, dx/dt = growth rate, $(1/x)dx/dt$ = specific growth rate, ds/dt = time rate of change of limiting substrate concentration, $\mu(s)$ = specific growth rate in absence of washout—assumed to be some function of the limiting substrate concentration, and $F(x, s)$ = a function describing the rate at which the limiting substrate is taken up by the organism.

Equation (1) is a simple balance equation (or statement of conservation),

$$\text{increase} = \text{growth} - \text{output}$$

where each term refers to a rate of increase of concentration of organisms by mass. Similarly, equation (2) is another simple balance equation of the form,

$$\text{increase} = \text{input} - \text{output} - \text{consumption}$$

where each term refers to a rate of change of limiting substrate concentration.

Growth rates are assumed to adjust themselves to changes in limiting substrate concentration instantaneously, and the ingoing medium is assumed to be instantaneously and uniformly dispersed throughout the culture vessel.

Spicer[9] has shown that very mild restrictions on the forms of $\mu(s)$ and $F(x, s)$ are necessary to insure that the system will return to a steady condition after small displacements from steady-state occur.

In order to make some progress with equations (1) and (2), it is customary to take

$$F(x, s) = x\mu(s)/Y \tag{3}$$

which assumes that, in the absence of washout, the rate of consumption of limiting substrate is equal to the growth rate divided by a yield constant Y. This constant is defined from conditions in a batch culture by

$$dx/dt = -Y\, ds/dt, \quad \text{or} \quad x = C - Ys \tag{4}$$

where C is some constant. Thus, for a finite period of growth, Y = weight of bacteria formed/weight of substrate used.

Multiplying (2) by Y and adding to (1), with substitution from (3) leads to a differential equation which may be solved to give

$$x = x_0 e^{-Dt} + Y(s_0 - s) \tag{5}$$

This rather simple result relates concentration of organisms by mass to limiting substrate concentration at all times in a one-vessel continuous fermentor, independently of the form assumed for the specific growth-rate dependence on substrate concentration.

Two special cases of equation (5) are of interest. First, setting $D = 0$, we obtain, for a batch culture,

$$x = x_0 + Y(s_0 - s) \tag{6}$$

a form of equation (4) with $C = x_0 + Ys_0$.

Second, setting $t = \infty$, when $D \neq 0$, and denoting steady-state values of x and s by $\bar{x}$ and $\bar{s}$, we have

$$\bar{s} + \bar{x}/Y = s_0 \tag{7}$$

This equation has the following simple interpretation:[8] at steady-state, the concentration of the substrate plus the mass concentration of the organism calculated as substrate equals the substrate concentration in the incoming medium.

Note that equations (6, 7) hold without the form of $\mu(s)$ being specified in equations (1, 2, 3). In order to have a stable steady-state, it is necessary to have[9]

$$d\mu(s)/ds > 0 \tag{8}$$

meaning that the specific growth-rate should be an increasing function of the limiting substrate concentration.

Various forms for $\mu(s)$ have been assumed.[2, 5, 6, 8] If we assume

$$\mu(s) = \mu_m s/(K + s) \tag{9}$$

where μ_m = maximum specific mass growth-rate constant for the organism and medium concerned, and K = saturation constant for the given organism and substrate, the value of s at which $\mu(s) = \mu_m/2$, and substitute for $\mu(s)$ according to (9) in equations (1, 2, 3), we obtain the differential equations for bacterial and substrate concentration in a continuous culture of the Monod type; namely,

$$dx/dt = x[\mu_m s/(K+s) - D] \tag{10}$$

$$ds/dt = D(s_0 - s) - (x/Y)[\mu_m s/(K+s)] \tag{11}$$

Usually, major interest is centred on the steady-state conditions resulting from equations (10, 11). To obtain these, set the left- and right-hand members of these equations equal to zero with the result that

$$\bar{s} = KD/(\mu_m - D) \tag{12}$$

$$\bar{x} = Y(s_0 - \bar{s}) \tag{13}$$

However, if one is interested in obtaining a description of the course of bacterial concentration from inoculation to achievement of the steady-state condition, one may solve equation (5) for s, substitute in equation (10), and make use of equations (12, 13) to obtain

$$dx/dt = (\mu_m - D)x[\bar{x} - x + x_0 e^{-Dt}]/[Y(K+\bar{s}) + \bar{x} - x + x_0 e^{-Dt}] \tag{14}$$

The implications of equation (14) are discussed more easily if a few changes in notation are made. Let $t' = \mu_m t$, $y = x/\bar{x}$—the bacterial concentration expressed as a fraction of the steady-state value, $y_0 = x_0/\bar{x}$, $W_1 = D/\mu_m$, $W_2 = K/s_0$, $W_3 = W_2/(1 - W_1 + W_1 W_2)$, then equation (14) becomes

$$dy/dt' = (1 - W_1)y[1 - y + y_0 e^{-W_1 t'}]/[W_3 + 1 - y + y_0 e^{-W_1 t'}] \tag{15}$$

According to equation (15), the relative bacterial concentration, y, may be plotted against $t' = \mu_m t$, and the shape of the growth curve will depend upon three parameters; namely,

y_0 = the initial inoculum expressed as a fraction of the steady-state concentration,

$W_1 = D/\mu_m$, the dilution rate expressed as a fraction of the maximum specific growth-rate constant,

$W_2 = K/s_0$, the saturation constant expressed as a fraction of the initial limiting substrate concentration.

Discussion of Equation (15)

In general, equation (15) cannot be solved for y in terms of elementary functions of t', y_0, W_1 and W_2. However, this can be done in the case of a batch culture.

1. *Batch Culture*

In this case, $D = 0$. Hence, $W_1 = 0$ and equation (15) becomes

$$dy/dt' = y[1+y_0-y]/[W_2+1+y_0-y] \tag{16}$$

In the customary treatment[1] of growth in a batch culture, it is usually assumed that, after a variable lag period, growth proceeds exponentially, with the organisms growing most of the time in substrate concentrations which are high compared to K.

In equation (16), an equivalent assumption is that W_2 is very close to 0 (K is a negligible fraction of s_0). Then

$$x \sim x_0 e^{\mu_m t'} \tag{17}$$

or the course of bacterial concentration proceeds approximately exponentially from an initial concentration of x_0 until the limiting substrate has been exhausted (from equation (12), $\bar{s} = 0$ when $D = 0$).

It is possible, however, to describe more precisely the effect of variation in values of $W_2 = K/s_0$ upon the course of bacterial concentration. Equation (16) may be solved to give

$$t' = \log (y/y_0) + W_4 \log [y/y_0(1+y_0-y)] \tag{18}$$

where $W_4 = W_2/(1+y_0)$, and the logarithms are to be computed to the base e.

In equation (18), one can assign values to y and easily calculate t' and thus make a graph of y against t' for each pair of values assigned to y_0 and W_2.

If one is interested in predicting the length of time, t_{99} for a batch culture to reach 99 per cent of the steady-state value, then one may write

$$t'_{99} = \log (0{\cdot}99/y_0) + W_4 \log [0{\cdot}99/y_0(0{\cdot}01+y_0)] \tag{19}$$

Graphs of t'_{99} against W_2 for a specified y_0 are straight lines, and for given values of y_0, K, and s_0, are easily used to find t_{99}.

For the experimental situation discussed in Herbert *et al.*,[1] where $\mu_m = 0{\cdot}85$/h, $K = 0{\cdot}0123$ g/l., $s_0 = 2{\cdot}5$ g/l., $W_2 = 0{\cdot}00492$, use of equation (18) shows that the resultant theoretical growth curve in a batch culture does not deviate significantly from an exponential growth law with a specific growth-rate constant equal to μ_m. Since low values of K relative to s_0 seem to be typical of many organisms and media, analysis of equation (18) shows that it follows from Monod's assumptions that theoretical bacterial growth is often well approximated by assuming that the process is in the logarithmic phase.

2. *Continuous Culture*

In the case of continuous culture, it does not seem possible to obtain a solution for y in terms of elementary functions of t', or *vice-versa*, as was done in equation (18) for a batch culture. Hence, equation (15) has to be handled, for particular values of the parameters, by some method of numerical solution.[4]

Inspection of equation (15) reveals that if $K = 0$, then the course of bacterial concentration follows an exponential law with

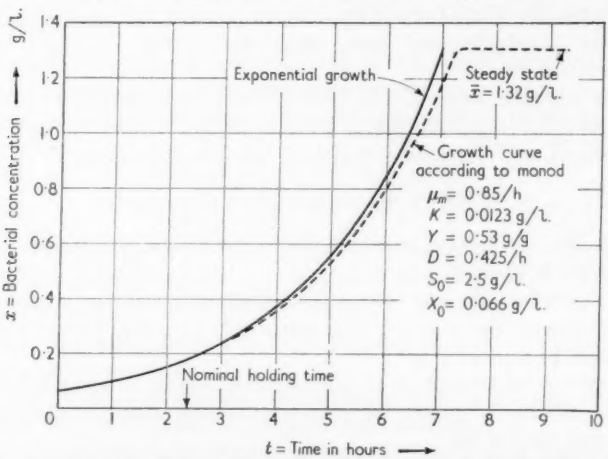

Fig. 1. Comparison of bacterial growth curve, for example from Herbert *et al.*,[1] with the case of exponential growth in a one vessel, continuous fermentor.

a specific growth-rate constant equal to $\mu_m - D$, a result which is also apparent from equation (10). Hence, when K is small compared to s_0 (or W_2 is close to 0), it may be adequate to assume that the above-mentioned exponential law holds to a good approximation, provided D is not too close to μ_m. Since W_2 is usually quite small in practice, the approximation will be very good for W_1 up to 0·9. Fig. 1 shows a comparison of the growth curve implied by equation (15), when $D = \mu_m/2$, with the approximating exponential curve for the organism and medium discussed by Herbert *et al.*[1]

Strictly, according to Monod's development, bacterial growth is never in a logarithmic phase, but may conveniently be assumed to be so for many cases of interest. Thus, the assumption[2] of a logarithmic growth region is equivalent to assuming that $K = 0$ in Monod's treatment.

Steady-state Criteria

Luedeking and Piret[2] have demonstrated that steady-state conditions are achieved in a single vessel continuous fermentor where toxic products of the fermentation limit the growth in a particular way. Perhaps it should be noted that the steady-state may be achieved under less restrictive assumptions and that the proof is implicit in the earlier work of Spicer[9] who demonstrates, for equations (1) and (2), that, if

$$\frac{\partial \mu(s)}{\partial s} \quad \text{and} \quad \frac{\partial F(x, s)}{\partial x}$$

are of the same sign and

$$\frac{\partial F(x, s)}{\partial s} \geqslant 0$$

these are sufficient conditions for stable equilibria.

Now, equations (21) and (22) of Luedeking and Piret[2] are special cases of our equations (1) and (2), hence it is simple to check the signs of the partial derivatives and establish that there is a stable steady-state.

Conclusions

In most experiences with constant-growth apparatus, the organisms used have been such that the saturation constant K in Monod's development is fairly small and is usually a small percentage of s_0, the incoming limiting substrate concentration. The evidence from Fig. 1 and equation (15) is that, when K/s_0 is small, bacterial concentration essentially increases exponentially with a specific growth-rate constant equal to $\mu_m - D$ (the maximum specific growth-rate constant minus the dilution rate) provided D/μ_m is not too close to 1. The approximation to exponential growth holds at least up to 99 per cent of the steady-state bacterial concentration.

The simultaneous rate equations of Luedeking and Piret for bacteria and toxic product are shown to be special cases of very general equations applicable to toxic or nutritional schemes. Further, the general requirements for stable equilibria are met.

Nomenclature

C	Constant of integration
D	Dilution rate
K	Saturation constant
s	Concentration of limiting substrate
s	Steady-state concentration of limiting substrate
t	Time
t'	$\mu_m t$
x	Concentration of organisms by mass
$\bar{x}$	Steady-state concentration of organisms by mass
y	Relative concentration of organisms by mass
Y	Yield constant
W_1–W_4	Defined in terms of μ_m, D, y_0, K, s_0
μ_m	Maximum specific growth-rate constant
$\mu(s)$	Specific growth-rate in absence of washout
$F(x, s)$	General function describing the rate at which the limiting substrate is taken up by the organism

Subscripts

0	Refers to initial conditions when $t = 0$
99	Refers to conditions when $y = 0{\cdot}99$

References

[1] Herbert, D., Elsworth, R. and Telling, R. C. The Continuous Culture of Bacteria; A Theoretical and Experimental Study. *J. gen. Microbiol.*, 14 (1956), 601

[2] Luedeking, R. and Piret, E. L. Transient and Steady States in Continuous Fermentation. Presented in part, 134th Meeting, Amer. Chem. Soc., Chicago, Sept. 1958. This Journal, 1 (1959), 431

[3] Maxon, W. D. Microbiological Process Report, Continuous Fermentation, A Discussion of Its Principles and Applications. *Appl. Microbiol.*, 3 (1955), 110

[4] Milne, W. E. *Numerical Solution of Differential Equations* (1953). New York; Wiley and Sons

[5] Monod, J. La Technique de Culture Continue; Théorie et Applications. *Ann. Inst. Pasteur*, 79 (1950), 330

[6] Novick, A. Growth of Bacteria. *Annu. Rev. Microbiol.*, 9 (1955), 97

[7] Novick, A. and Szilard, L. Experiments with the Chemostat on Spontaneous Mutations of Bacteria. *Proc. nat. Acad. Sci., Wash.*, 36 (1950), 708

[8] Powell, E. O. Criteria for the Growth of Contaminants and Mutants in Continuous Culture. *J. gen. Microbiol.*, 18 (1958), 259

[9] Spicer, C. C. The Theory of Bacterial Constant Growth Apparatus. *Biometrics*, 11 (1955), 225

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Continuous Antibiotic Fermentation—Design of a 20 litre, Single-stage Pilot Plant and Trials with Two Contrasting Processes*

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Summary. The feasibility of producing antibiotics by single-stage continuous fermentation was explored by means of a specially designed pilot plant. Both the chloramphenicol and penicillin processes appeared adaptable to such an operation. At dilution rates of 1·0 and 0·5 volume changes per day respectively, yields of from $\frac{1}{4}$ to $\frac{1}{2}$ of the maxima obtained in batch operation were maintained in the steady-state for more than 2 weeks.

Introduction

Although the potential advantages of continuous fermentation are widely recognized and the theoretical basis is reasonably established, industrial use of the operation has been restricted almost entirely to yeast and yeast products, vinegar, and sewage treatment processes, all of which yield bulk products and depend for their advantageous operation on a highly restrictive growth situation.[1,2] Future applications of continuous culture, as judged by current trends in research and development,[3, 4] will probably extend into the production of fine chemicals and into fermentations where culture purity is much more precarious.

One such application of considerable potential importance is in antibiotic production. Since the open literature carried many but only brief notes of progress in this area (see later), we undertook a study to determine the feasibility of continuous antibiotic fermentation. This report describes the design of a 20 l.,

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single-stage pilot plant and the outcome of fermentation trials of two contrasting antibiotic processes, chloramphenicol and penicillin.

Pilot Plant Design and Operation

We sought to design a fermentation unit which would be as simple as possible and potentially capable of scale-up to existing production equipment. The size, 20 l. working capacity, was consistent with usual practice and with the requirements of sampling and adequate feed pumping. The materials of construction were chosen with a view not only to economy but to their specific suitability. For instance, black iron pipe was used for air and steam lines, but the fermentor vent line was constructed of stainless steel because the surging of liquid within this line might otherwise build up high concentrations of iron in the culture. The fermentor and sterilizer vessels were made of Inconel-clad stainless steel. Feed lines were gum-rubber tubing.

A wide-angle view of the installation at one stage of its design is shown in Fig. 1, and a schematic diagram in Fig. 2. The feed

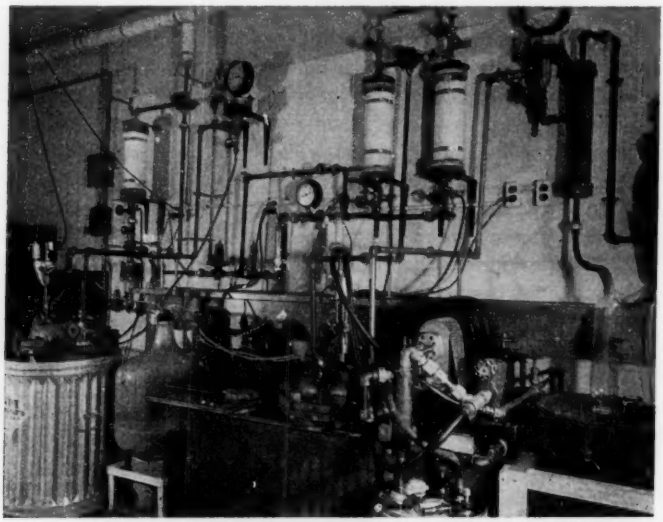

Fig. 1. Wide-angle view of pilot plant installation

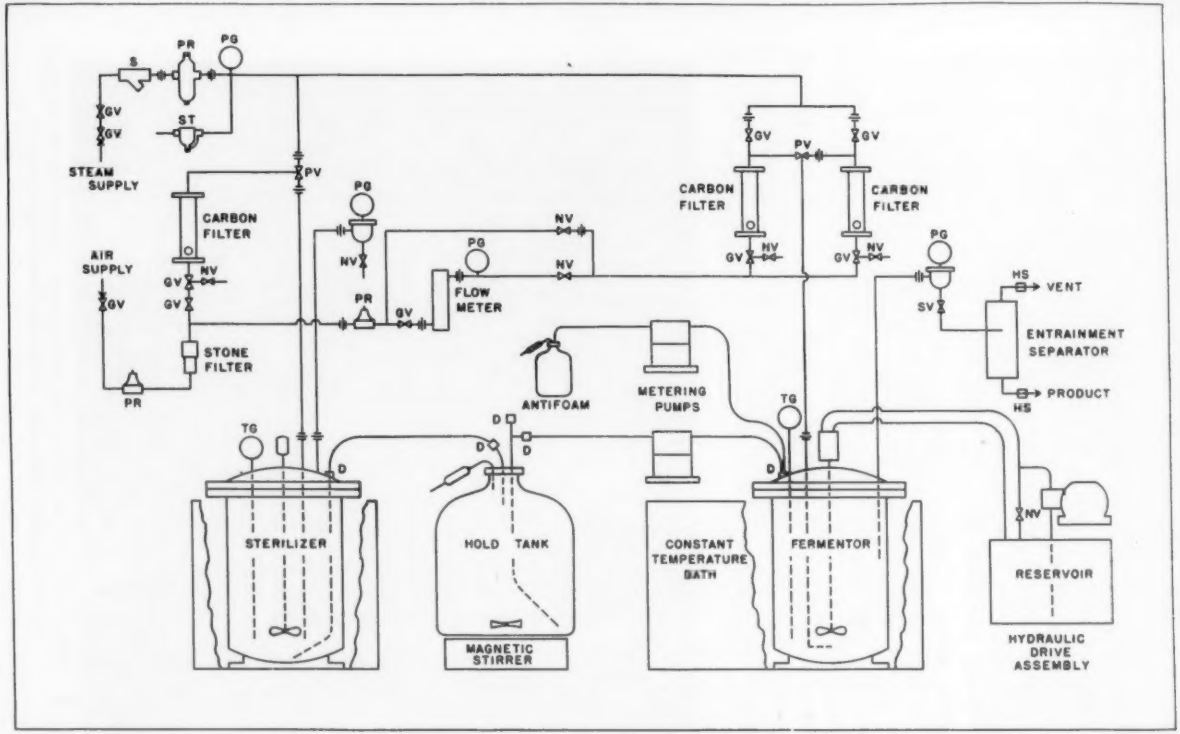

Fig. 2. Schematic diagram of pilot plant

GV, gate valve; NV, needle-valve; SV, Saunders-type valve; PV, plug valve; PR, pressure regulator; PG, pressure gauge; TG, temperature gauge; ST, steam trap; S, strainer; HS, heat seal; D, diaphragm

system was operated by first preparing a batch of medium and cooking it under pressure in the sterilizer vessel. The medium then was transferred by means of differential air pressure to the separately sterilized hold tank, a magnetically stirred, 13 gal Pyrex carboy. From this reservoir, the sterile medium was continuously metered into the fermentor by a peristaltic-action Sigma pump. Large hypodermic needles in the connecting feed lines and self-sealing rubber diaphragms fitted to the vessels permitted isolating the hold tank and fermentor from the sterilizer and also replacing the feed lines if necessary during operation.

The air supply to the fermentor was successively stone-filtered, metered, and sterilized by passage through an activated-carbon filter. Duplicate insulated filters (Fig. 3) were piped so as to

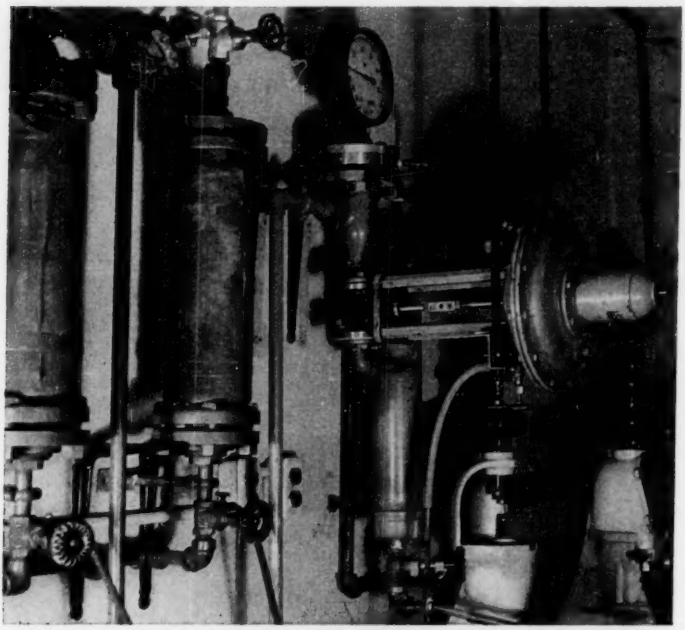

Fig. 3. Close-up view of air filters (left), back-pressure regulating unit (centre), and entrainment separator (behind and below regulator)

allow alternate operation and steam sterilization on a 3-day cycle. The problem of occasional combustion of the carbon packing was solved by adding stainless steel wool to dissipate localized heat and by inspecting the packing through plugs in the bottom of the filters. The sterile air was passed into the contents of the fermentor through a $\frac{3}{8}$ in. stainless steel pipe opening $\frac{1}{2}$ in. below the centre of the impeller.

The fermentor vessel was fitted with a diaphragm adapter at the top for inserting the feed lines, and also near the bottom for sampling the contents by means of a sterile hypodermic syringe and needle. The contents of the fermentor were kept at constant temperature by means of an electrical thermoregulator and saber-heating unit acting against a constant flow of cold water in the water bath. A hydraulically driven turbine positioned on top of the fermentor drove the agitator shaft and conventional four-bladed impeller. The agitator shaft entered the vessel through a stuffing box packed alternately with $\frac{1}{4}$ in. teflon-impregnated asbestos rings and $\frac{1}{2}$ in. teflon spacers; mechanical seals were also attempted but proved less satisfactory. With the possibility of agitator speeds up to 700 rev/min and with the fermentor equipped with four vertical baffles, fully adequate agitation could be attained.

Both air and product were withdrawn through a pipe that extended into the fermentor to the liquid level. Maintenance of a constant liquid level in the fermentor with such an air-lift system required close control of foaming and this was accomplished with the continuous addition of antifoam agent. A separate sterile reservoir and continuous feed line for antifoam agent were necessary in the penicillin process, but a stable silicone emulsion could be incorporated into the medium in the chloramphenicol process. A spring-loaded, back-pressure regulating valve and a product–air separator were located in the effluent system and are shown in Fig. 3. The pressure regulator maintained a positive pressure of about 5 lb/in^2 gauge in the fermentor independent of the rate of flow of medium or air. Electric heating tapes used as heat seals were located on the effluent air and product pipes to prevent possible back-growth of contaminating micro-organisms along the pipe walls.

Chloramphenicol Fermentation

Chloramphenicol appears to be ideally adapted to continuous fermentation in a single-stage unit, because the batch fermentation exhibits parallel growth and antibiotic formation and, in addition, is favourably characterized by only moderate pH changes, a fluid cell suspension, wide-spectrum effectiveness against contamination, and stable product. As potential disadvantages, the hyphae of the chloramphenicol-producing *Streptomyces venezuelae* tend to fragment and lyse during the latter stages of the batch growth cycle, and the organism is understood to have a moderately high frequency of reversion to mutants with low synthetic ability. Details of the batch chloramphenicol process have been published.[23, 24] To our knowledge, continuous fermentation of this antibiotic has not been attempted, although there have been brief reports or patents on continuous fermentation of streptomycin,[4–6] subtilin,[11] and penicillin (see below); these are discussed more fully elsewhere.[12]

Assay procedures. Product samples were withdrawn directly from the fermentor and frozen until a number of assays for antibiotic content could be made together. Chloramphenicol was determined by the method of Glazko *et al.*[13] The aromatic nitro group of chloramphenicol was quantitatively reduced to a primary amine, which was then determined colorimetrically by the standard Bratton–Marshall diazotization procedure. Correction was made for non-specific aryl amines, and a standard curve was included with each set of determinations. The method measures both the active antibiotic and inactive degradation products; the latter, however, are believed to be present only in a very low concentration, and in any event should remain relatively constant in amount during continuous fermentation.

The pH and percentage of total solids in the crude product were estimated at the time of sampling. The solids content was determined on a volume basis after centrifuging a 10 ml sample for 10 min at $2000 \times g$.

Inoculum preparation. A culture of *Str. venezuelae* strain 05072 was obtained from Parke, Davis and Company; this strain can produce about 400 μg of chloramphenicol per ml. Soil stocks of the organism were prepared from mature spores after growth

on Anderson's sporulation agar.[7] When a fermentation was begun, the spore stock was inoculated into 100 ml of chloramphenicol medium II,[7] modified as shown in Table I, in a 250 ml Erlenmeyer flask. This was incubated on a rotary shaker at 28°C for 48 h and then used as inoculum for a larger 600 ml culture in a 2 l. flask. Three such flask cultures (7·5 volume–per cent) were used to inoculate the fermentor.

Batch chloramphenicol fermentation. A series of batch fermentations were conducted to establish a reference picture in our equipment and with our operating procedures. The fermentor contained 20 l. of the modified chloramphenicol medium (Table I)

Table I. Composition of fermentation mediums

Chloramphenicol medium*		Penicillin inoculum medium†		Penicillin fermentation medium†	
Ingredients	g/l.	Ingredients	g/l.	Ingredients	g/l.
Glycerol (ml/l.)	10	Glucose	20	Lactose	55
Brewers yeast	15	Corn-steep solids (dry weight)	10	Glucose	5
Butanol-acetone fermentation residue	5	$CaCO_3$ (total)	5	Corn-steep solids (dry weight)	45
NaCl	5	$ZnSO_4$	0·1	$CaCO_3$	5
$CaCO_3$	1	K_2HPO_4	0·5	$ZnSO_4$	0·05
NaOH to pH 7·3–7·4		$NaNO_3$	1	KH_2PO_4	0·5
q.s. tap water		$MgSO_4$	0·1	Na_2SO_4	0·5
		q.s. tap water		Potassium phenyl acetate	0·5
				Crude lard oil	3
				q.s. tap water	

* Provided by Parke, Davis and Company.
† Provided by Eli Lilly and Company.

with 1 ml of General Electric No. 60 silicone–emulsion defoamer; air was supplied at a rate of $\frac{3}{4}$ volumes per volume of medium per min; the agitator was driven at a speed of 250 rev/min, and the temperature was regulated at 28°C.

Results of a representative batch chloramphenicol fermentation under these conditions are shown in Fig. 4. The maximum yield of 400 μg/ml in 4 days is relatively good for this strain. This particular experiment also was selected to demonstrate that even the early appearance of contamination sometimes has no apparent effect on chloramphenicol formation, a useful

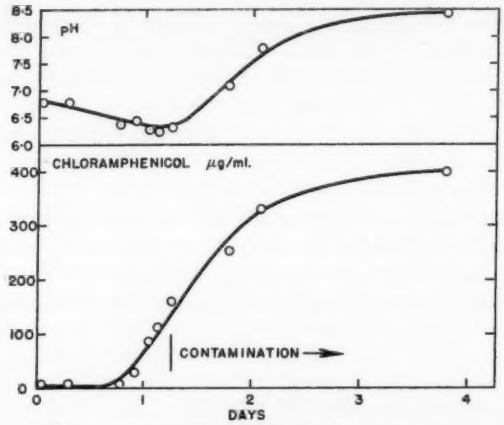

Fig. 4. Batch chloramphenicol fermentation

characteristic for continuous fermentation. However, the abnormal pH changes may have been caused by the contamination.

Continuous chloramphenicol fermentation. A continuous fermentation was begun in the same manner as a batch fermentation, allowed to progress for 24 h, and then put on continuous feed with the same medium. Results of such a trial are shown in Fig. 5.

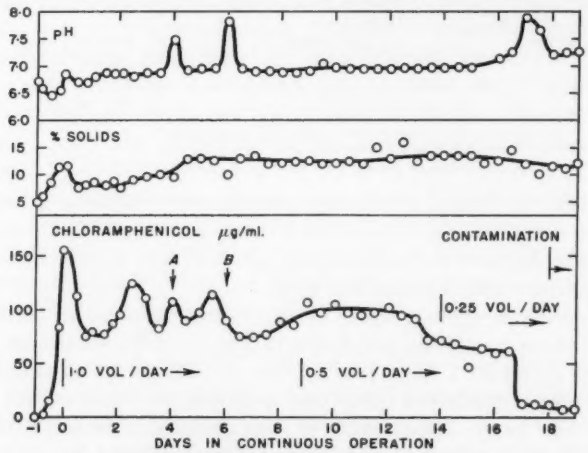

Fig. 5. Continuous chloramphenicol fermentation

It was found that a dilution rate of 1·0 volume changes per day brought an initial drop from the higher batch level and then allowed maintenance of about one-quarter of the maximum batch yield. In three confirmatory experiments of shorter duration with the same dilution rate, yield levels again dropped initially and then were maintained at between 80 and 110 μg/ml. As shown in Fig. 5, lower feed rates tended, if anything, to reduce the yield, although the pH and solids were relatively unaffected. The yield fluctuations observed in this and other continuous fermentations usually could be traced to some operational deviation and did not appear to be inherent in the method. For example, at points *A* and *B* on Fig. 5, the feed pump was inadvertently stopped for 5 and 10 h, respectively. The appearance of contamination after 18½ days of continuous operation apparently had caused the yield to drop, and the experiment was terminated after 20 days.

The question was raised whether the drop in chloramphenicol production might be due rather to 'strain degeneration', especially since a lower yield level unpredictably accompanied the lowering of the feed rate in the third week. Accordingly, the organism was isolated from samples taken at the start of continuous operation as well as after 17 days in this experiment, and after 8 days in another experiment. Each of these cultures was then used to inoculate duplicate shaker flasks of medium and the resulting batch fermentations were analysed daily over a 10-day period. These results are given in Table II. Although the fermentations

Table II. Batch fermentation tests* of inoculums isolated initially and terminally from continuous chloramphenicol fermentations

	Inoculum from Exp. A						Inoculum from Exp. B (Fig. 5)					
	0 days			8 days			0 days			17 days		
Hours of incubation	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.
48	7·05	5	3	6·95	5	2	7·00	4	0	7·00	5	3
72	7·20	7	18	7·18	7	25	7·15	5	0	7·20	5	4
96	7·55	8	36	7·40	9	37	7·40	7	16	7·35	7	17
120	7·75	10	47	7·65	9	45	7·65	7	24	7·50	8	35
144	7·85	11	50	7·90	10	55	7·90	8	40	7·85	8	44
168	8·10	10	58	7·95	12	63	8·00	8	44	7·95	8	50
192	8·15	11	68	8·00	15	72	8·15	8	48	8·05	9	51
216	8·30	13	68	8·20	15	76	8·25	8	47	8·30	10	50
240	8·45	14	67	8·40	15	72	8·40	8	37	8·40	10	45

* Values given are averages of duplicate determinations.

were in all cases more protracted and gave lower yields than usual, differences attributable to 'strain degeneration' from continuous culture were not apparent.

Penicillin Fermentation

In contrast to chloramphenicol, penicillin characteristically is formed after mycelial growth has approached its maximum, suggesting that the usual batch cycle is composed of two phases.[32] Consequently, penicillin production under steady-state conditions should occur to best advantage in at least a two-stage system in which the growth and production phases could be separated and possibly enhanced individually. At best, single-stage continuous penicillin fermentation should represent only a low resultant of the two phases balanced at the steady-state. Although this outlook has generally prevailed, a recent note by Somerson and Nunheimer[30] indicated that factors in the medium, rather than the mould mycelium, are responsible for the limitation of penicillin biosynthesis early and late in the batch cycle; thus, continuous penicillin fermentation in a single-stage system might be expected to be successful.

In further contrast to the chloramphenicol batch process, the penicillin fermentation is characterized by greater pH changes, a thick mycelial suspension, antibiotic activity primarily against Gram-positive bacteria, and a product that is unstable to both temperature and acid as well as to the penicillinase often produced by contaminating bacteria. The *Penicillium* mycelium tends neither to lyse nor to form spores in deep liquid culture.

A considerable amount of literature exists on the penicillin batch process,[32] but continuous fermentations have been reported only in brief notes or as patents. Abraham[1] and subsequently others[14, 19, 20, 31] have devised systems for continuously passing fresh medium beneath surface cultures or[17] inside a Cellophane or porcelain tube on which the organism was grown. Attempts at single-stage continuous submerged fermentation have been made in shaving-packed generators[2, 28] and in more conventional 30 l. fermentors,[16, 27] in which yields of 500 to 800 units per ml were maintained for 5 days. Several semi-continuous operations have been described in which a portion of a completed

batch is retained in the fermentor as starter for a new cycle.[10, 26, 33] A patent has been awarded for a two-stage continuous penicillin process in which overflow from a primary propagator is fed to a secondary fermentor.[18]

Assay procedures. The procedures for sampling and for determining pH and percentage of solids in the penicillin fermentations were the same as in the chloramphenicol process. Total carbohydrates were determined by the standard anthrone reaction. Penicillin was assayed by a paper-disc, zone-inhibition test similar to that recommended by the U.S. Food and Drug Administration.[9] Commercial dehydrated media (Difco) with strain H of *Staph. aureus* (obtained from Eli Lilly and Company) as the test organism were used in the assay procedure.

Inoculum preparation. A lyophilized spore suspension of *Penicillium chrysogenum* strain N–329 was obtained from Eli Lilly and Company; this strain can produce about 2,000 units per ml but has a comparatively protracted fermentation period. Slant spore stocks of the mould were prepared by inoculating the lyophilized spores on Moyer's sporulation medium,[21] incubating the cultures 8 days at 27 °C, and storing them in the refrigerator until needed. To begin a fermentation, spore suspension from one such tube was inoculated into 2 l. Erlenmeyer flasks containing 500 ml of Czapek–Dox corn-steep liquor medium[25] modified for inoculum growth as shown in Table I. After incubation at 25 to 28 °C on a shaker for 40 to 48 h, three such cultures (7·5 volume–per cent) were used to inoculate the fermentor.

Batch penicillin fermentation. As with the chloramphenicol experiments, a series of batch penicillin fermentations were conducted initially. The fermentation medium used (Table I) was typical of the production media reported in the literature, and included dried corn-steep liquor solids, potassium phenyl acetate, and lard oil. Air was supplied at a rate of 1·0 volume per volume of medium per min, the agitator was operated at a speed of 550 rev/min, and the temperature was regulated at 26 °C.

Results of a representative batch penicillin fermentation under these conditions are shown in Fig. 6. The maximum yield of 1,800 units per ml in 7 days is representative for this strain, but the usual appearance of mycelial growth before the penicillin formation was not observed and the pH curve is atypical.

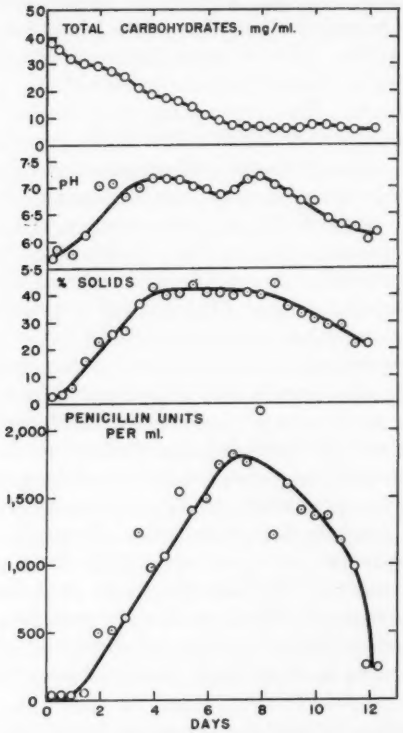

Fig. 6. Batch penicillin fermentation

Continuous penicillin fermentation. A continuous fermentation was begun in the same manner as a batch fermentation, allowed to progress for 48 h, and then put on continuous feed with the same medium but with the lard oil fed separately. The first continuous run was made with a dilution rate of 1·0 volume changes per day, and as shown by the results in Fig. 7, both the mycelium and the penicillin level initially rose but subsequently were washed out after $4\frac{1}{2}$ days of continuous operation. At point *A* on Fig. 7, the feed was stopped for 6 h and then was resumed at point *B*; at point *C* the feed again was stopped for the duration

of the run. The rises in antibiotic level that occurred at the points when the fermentation was allowed to proceed batch-wise indicated that the mould had not lost its ability to synthesize penicillin during the $4\frac{1}{2}$ days of continuous culture. Two additional problems presented themselves in this first experiment: a heavy growth of eventually sporulating mycelium

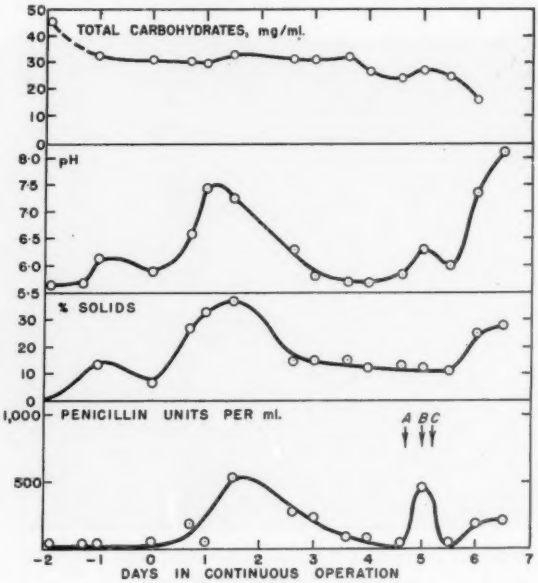

Fig. 7. Continuous penicillin fermentation at 1·0 volume changes per day

accumulated on the walls and baffles inside the fermentor (Fig. 8), and the mycelium in the effluent line after $4\frac{1}{2}$ days plugged the back-pressure regulating valve. The first problem of mycelium accumulating inside the fermentor was never satisfactorily resolved; it appears to be an inherent limitation of continuous operation. The second problem was circumvented, or at least postponed, by substituting a vertically mounted Grinnell 'straightway' diaphragm valve for the conventional Saunders type in the pressure regulating unit.

With this change in equipment and reduction of the medium feed rate to 0·5 volume changes per day, a second continuous penicillin fermentation (Fig. 9) was maintained successfully for 13 days, with a yield level between 400 and 1,000 units per ml. Contamination occurred at 13 days, at which time the penicillin yield dropped and the run was terminated. Although the pH remained relatively steady during continuous operation, there

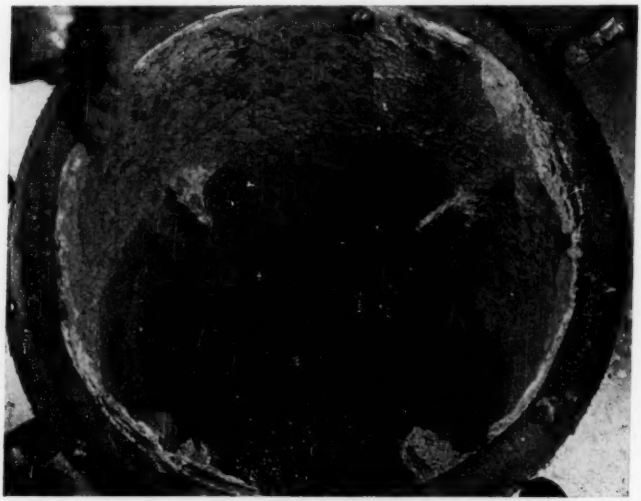

Fig. 8. View of accumulated mycelium in fermentor after 9 days of continuous penicillin fermentation

was considerable variation in the antibiotic level. As before, such fluctuations could usually be traced to an operational variable. For example, at points *A* and *B* on Fig. 9, the fermentor contents were observed to be foaming excessively; extra addition of antifoam agent was accompanied by an increased antibiotic level.

As shown in Fig. 10, a confirmatory run under the same conditions again reached and maintained for 3 days a penicillin level of 1,000 units per ml. However, another operational variable again apparently caused reduced yields twice during the 8-day

period of these operating conditions; at points *A* and *B* on Fig. 10, a valve was observed to be operating defectively and allowing steam condensate to enter the fermentor. In both this and the preceding run, the level of total carbohydrates steadily and unexplainably diminished during continuous fermentation. After

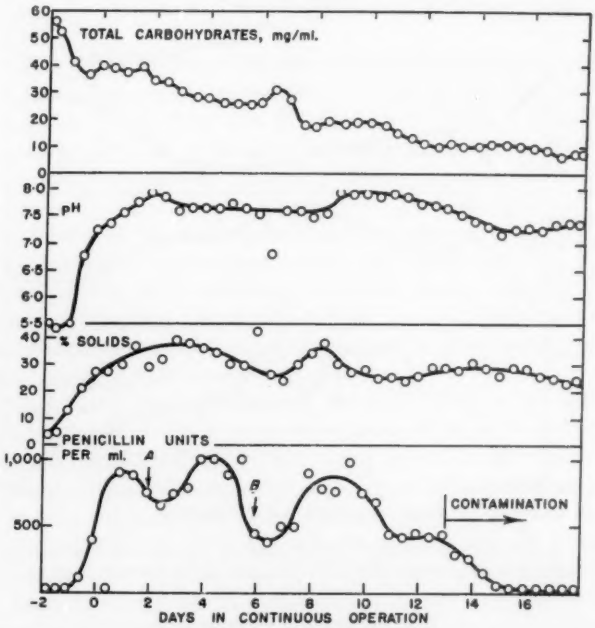

Fig. 9. Continuous penicillin fermentation at 0·5 volume changes per day

8 days, including recovery of the fermentation from the effects of the steam condensate, the medium being fed was changed so that 1·44 per cent glucose replaced the lactose originally employed. At the medium feed rate used, the glucose feed rate was 0·03 per cent per h. The use of glucose as a carbohydrate source at this feed rate was reported by Soltero and Johnson[29] to give better penicillin biosynthesis in batch fermentations. In our continuous operation, however, this change

was associated with a gradual reduction in penicillin, solids, and carbohydrates and with a rise in pH.

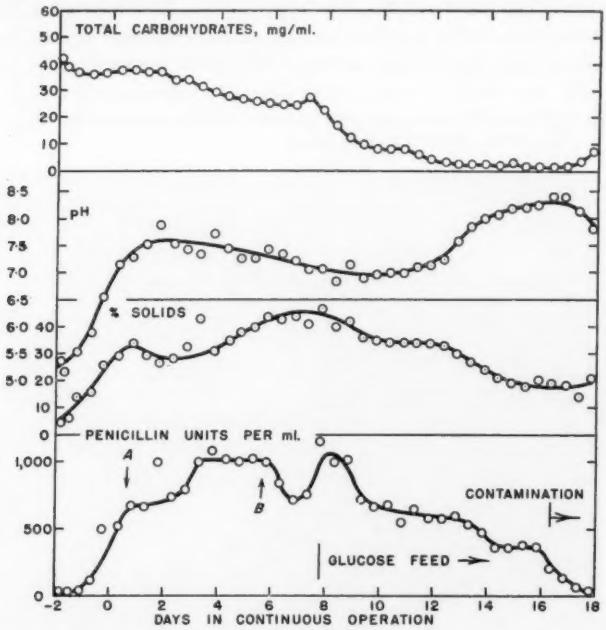

Fig. 10. Continuous penicillin fermentation at 0·5 volume changes per day, initially with lactose and then with glucose as the carbohydrate

Discussion and Conclusions

It appears that both chloramphenicol and penicillin can be produced by single-stage continuous fermentation processes. Using dilution rates of 1·0 and 0·5 volume changes per day, respectively, we were able to maintain both fermentations in the steady-state for periods in excess of 2 weeks with yields from $\frac{1}{4}$ to $\frac{1}{2}$ of the maxima we obtained in batch operation. In normal industrial practice, of course, production batches are harvested before the maximum is reached. Moreover, if the continuous yields are put on a daily production basis over a two-week operating period, they become roughly equal to or, in the case of

penicillin, almost twice those achieved over the same period by batch operation with its characteristic shutdowns. It seems likely that further development of the continuous process could make its daily yield considerably more favourable. However, the relative practicability of the two types of operations depends on a number of features other than yields, so that further comparisons on the basis of the limited data so far available are not worth while.

A prominent feature of both of the continuous fermentations was the fluctuation, particularly in antibiotic level, with the passage of time. Finn and Wilson[8] have reported that the continuous growth of yeast may induce a sinusoidal cycling of the population, which will normally decay exponentially. In our continuous antibiotic fermentations, the oscillations usually could be traced to some operational deviation and did not appear to be inherent in the operation. The occurrence of plateaux during periods of several days in some of the experiments suggested that better controls should permit the attainment of true steady-state fermentations with antibiotic production levelling at the peaks of the observed oscillations.

The finding that continuous fermentations of penicillin required a lower dilution rate than those of chloramphenicol was expected, since moulds usually grow more slowly than actinomycetes. Totally unexpected was the observation that a lower continuous dilution rate lowered rather than raised the chloramphenicol yield. Since this occurred at the end of a run and since replacement of the original population with a mutant strain has been predicted to be inevitable in continuous culture,[22] the lowered yield level might have been due to 'strain degeneration' rather than to the lowered feed rate. An experiment to test this idea, however, indicated otherwise. Moreover, the result could not be traced to contamination or to the accumulation of toxic factors, since a return to a batch operation resulted in a rise in the yield. Since it was not accompanied by reduced mycelial solids, the reduced yield apparently was the result of a factor limiting only the biosynthesis of the antibiotic. The phenomenon requires further study.

The accumulation of *Penicillium* mycelium on surfaces inside the fermentor and within pipes and valves may prove to be an

inherent and ultimate limitation of continuous production of penicillin. The situation in pipes and valves may be circumvented and postponed by appropriate modification of equipment. Conceivably, the mycelium retained within the fermentor may even contribute significantly to the steady-state yield level. Eventually, however, the resulting congestion will force cessation of the continuous operation, unless some method can be devised to prevent it.

Contamination with extraneous micro-organisms was common to all of the continuous fermentations conducted and represents another important limiting factor in successful continuous operation. Its control ultimately depends on refinements in equipment and handling, but certainly is possible. Although we operated aseptically for periods in excess of 2 weeks, other groups have conducted continuous bacterial cultures in a similar pilot plant for periods of several months.[15] In almost every instance, we could trace contamination to the failure of some part of the equipment. Correction of such sources was and continues to be an important part of the development, e.g. replacing a fragile mechanical seal on the agitator shaft with a stuffing box. The larger, permanent equipment, accumulated experience, and possible integration of continuous sterilization with continuous fermentation should obviate many of the present problems of contamination if continuous fermentation becomes adopted in the fermentation industry.

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Mrs. Jo-Ann Cherry, Mr. D. M. Gallup, and Miss Sara S. DeLong conducted the analyses.

References

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A. and Florey, H. W. *Lancet*, 2 (1941), 177

[2] Clifton, C. E. *Science*, 98 (1943), 69

[3] *Continuous Cultivation of Microorganisms, A Symposium* (1958), Prague; Czechoslovak Academy of Sciences

[4] Continuous Culture Methods and Their Application. In *Recent Progress in Microbiology*, Symposia of VIIth Intern. Congr. Microbiol. (1958). Uppsala, Sweden; Almquist and Wiksells

[5] Distillers Co. Ltd., Ashton, H. W. and Holgate, J. (to Distillers Co. Ltd.) Brit. Pat. 660,145 (1951)
[6] Distillers Co. Ltd., Jackson, C. J. and Milner, J. (to Distillers Co. Ltd.) Brit. Pat. 644,078 (1950)
[7] Ehrlich, J., Anderson, L. E., Coffey, G. L. and Gottlieb, D. *Antibiotics and Chemotherapy*, 3 (1953), 1141
[8] Finn, R. K. and Wilson, R. E. *J. Agr. Food Chem*, 2 (1954), 66
[9] Food and Drug Administration, *Compilation of Regulations for Tests and Methods of Assay of Antibiotic Drugs*, Vol. I, Section 141a 1h (1954). U.S. Department of Health, Education and Welfare, Washington, D.C.
[10] Foster, J. W. and McDaniel, L. E. U.S Pat. 2,584,009 (1952)
[11] Garibaldi, J. A. and Feeney, R. E. *Industr. Engng. Chem.* (*Industr.*), 41 (1949), 432
[12] Gerhardt, P. and Bartlett, M. C. *Advanc. Appl. Microbiol.*, 1 (1959), 215
[13] Glazko, A. J., Wolf, L. M. and Dill, W. A. *Arch. Biochem.* (Biophys.), 23 (1949), 411
[14] Haller, G. U.S. Pat. 2,519,902 (1950)
[15] Herbert, D., Elsworth, R. and Telling, R. C. *J. gen. Microbiol.*, 14 (1956), 601
[16] Kolachov, P. J. and Schneider, W. C. U.S. Pat. 2,609,327 (1952)
[17] Lewis, R. W. and Lucas, E. H. *Science*, 101 (1945), 364
[18] Liebman, A. J. and de Becze, G. T. U.S. Pat. 2,524,200 (1950)
[19] Lilly, C. H. U.S. Pat. 1,936,983 (1933)
[20] Moor, W. A. *Science*, 102 (1945), 594
[21] Moyer, A. J. and Coghill, R. D. *J. Bact.*, 51 (1946), 57
[22] Novick, A. *Annu. Rev. Microbiol.*, 9 (1955), 97
[23] Olive, T. R. *Chem. Eng.*, 56 (1949), 107, 172
[24] Oyaas, J. E., Ehrlich, J. and Smith, R. M. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 1775
[25] Raper, K. B. and Thom, C. *A Manual of the Penicillia* (1949). Baltimore; Williams and Wilkins Co.
[26] Schenley Industries, Inc. French Pat. 1,018,459 (1953)
[27] Schneider, W. C., Andreasen, A. A. and Kolachov, P. J. 114th Meeting, Amer. Chem. Soc., p. 16A (1948). Portland, Ore.
[28] Schwarze, W. Swiss Pat. 279,098 (1952)
[29] Soltero, F. V. and Johnson, M. J. *Appl. Microbiol.*, 2 (1954), 41
[30] Somerson, N. L. and Nunheimer, T. D. *Bacteriol. Proc.* (Soc. Amer. Bacteriologists) (1958), p. 12
[31] Stice, E. and Pratt, R. *Science*, 103 (1946), 535
[32] Sylvester, J. C. and Coghill, R. D. *Industrial Fermentations*, Vol. II (1954). New York; Chemical Publishing Co., Inc.
[33] Wakaki, S., Ishida, T., Yamaguchi, Y., Mizuhara, K., Masuda, K. and Kondo, Y. *J. Antibiotics* (*Japan*), 5 (1952), 622

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Continuous Streptomycin Fermentation

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Summary. A pilot plant unit for continuous cultivation of hyphae-forming micro-organisms, providing for a uniform flow velocity in fermentations on suspension media with a massive growth of mycelium, is described. For the maintenance of aseptic conditions, over-pressure of air is maintained in the whole equipment. This equipment has been used for studies of the continuous biosynthesis of streptomycin. A three-stage fermentation proved the most advantageous. The first stage serves for multiplication of the inoculum; the second and third for the formation of the antibiotic. The system was maintained for 300–400 h with yields amounting to 2,000–2,500 u. of streptomycin/ml without any signs of contamination or degeneration of the gr owing culture.

Introduction

Continuous cultivation of micro-organisms is gaining ever-increasing importance in microbiology for the solution of both theoretical and practical problems, due to its numerous advantages compared with batch cultivation, as was pointed out by Novick.[1]

The basic theory of continuous cultivation was first published by Monod,[2] and Novick and Szilard,[3, 4] and further developed by numerous authors.[5–9] There exist two basic types of continuous cultivation method and two basic types of cultivation equipment. One of these is called 'chemostat',[3] or 'bactogen',[2] the other 'turbidostat'.[10] In the first method fresh medium is added at constant velocity to the cultivation vessel in which a constant volume of medium is maintained. The velocity of growth of the culture is limited by lowering the concentration of one of the basic nutrients, the other nutrients being in excess. In the second method, the equilibrium state is reached by increased velocity of inflow for an increasing number of micro-organisms in the vessel

and by decreased inflow for a decreasing number of micro-organisms in the vessel. The other types of equipment which have been described so far are more or less modifications of the two basic systems, e.g. the auxanometer,[11] the breeder,[12] and a number of others.[5, 13–15]

The method of continuous cultivation of micro-organisms has already been used in a number of varied applications: for the multiplication of a large number of pathogenic micro-organisms,[16, 17] in the field of microbial genetics in a study of mutation conditions,[4, 18] for the selection of bacterial cultures,[10] and for the study of enzyme formation.[2, 19, 20]

Simultaneously with the solution of theoretical questions, possibilities of application of the continuous method in various branches of the fermentation industry were considered. One of the very first publications on continuous cultivation[21] was concerned with the possibility of continuous production of lactic acid. Continuous propagation of baker's yeast,[22] of fodder yeast,[23, 24] of alcohol,[25, 26] and of butanol[27] were also objects of investigation. In the construction of equipment for continuous cultivation special emphasis has been laid upon the maintenance of a uniform flow of medium. Various devices for this purpose have been described: the principle of the Mariotte bottle,[13, 15, 28] rotameters[8, 29] and various types of pumps.[6, 30] In all the types of apparatus described so far, however, only clear, non-suspension type media may be used.

In industrial-scale production of antibiotics rich suspension-type media are used almost exclusively in order to secure maximum yields. One of the basic conditions of successful production of antibiotics is the maintenance of strictly aseptic cultivation conditions, achieved by an over-pressure of air in the whole equipment; this constituting a substantial difference from the production of baker's yeast, fodder yeast, and alcohol. A further factor, influencing profoundly the production of antibiotics, is aeration and agitation of the fermented liquid. During intense aeration, especially of suspension media, strong foam formation often takes place and defoaming is very difficult.

In this paper we describe an apparatus for the continuous cultivation of hyphae-forming micro-organisms, providing a uniform flow velocity even in fermentations on suspension media

with a powerful growth of mycelium. For aseptic cultivation conditions, air over-pressure is maintained in the whole equipment.

General Description

The equipment is shown schematically in Fig. 1. It consists of a tank (S) of 80 l. working capacity in which the medium is prepared and sterilized. From this tank, sterile medium is let

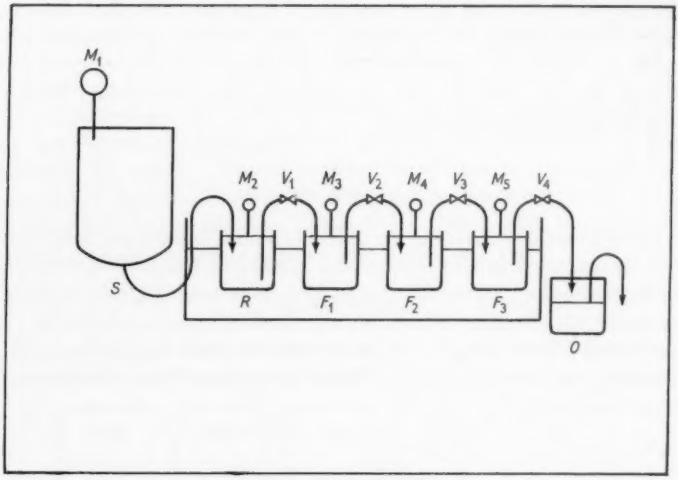

Fig. 1. Continuous culture plant

S—sterilizing vessel
R—reservoir
F_1–F_3—fermentors
O—collector
V_1–V_4 electromagnetic valves
M_1–M_5 manometers

out into a small tank (R) of 20 l. capacity, serving as an intermediate reservoir for medium while it is being prepared and sterilized in the large tank. From the small tank medium flows into one, two or three little tanks (F_1, F_2, F_3) mutually connected, of 5–15 l. capacity, in which the cultivation proper takes place, in one, two or three stages. The fermented medium is collected in the collector (O).

The velocity of flow of medium is regulated by electromagnetic

valves (V_1–V_4). The valves are governed by two adjustable relay switches, one of which determines the opening time of the valve, the other the frequency of medium injections. For aseptic conditions of cultivation air over-pressure is maintained in the whole equipment and measured by manometers (M_1–M_5). Air over-pressure decreases from the value of 0·8 atm in tank S to 0·2 atm in the collector O. Gradual decrease of over-pressure in the sequence of vessels is necessary for functioning of the valves and for the regulation of flow velocity. For the maintenance of an equilibrium state in the growing culture the outflow velocity has to be equal to the inflow velocity. This involves the following items of equipment: the cultivation vessel for the preparation and sterilization of the medium, the reservoir, the electromagnetic valves, the relay switch, piping and collector. A detailed description of all these parts follows.

Cultivation Vessel

As fermentation vessels we used standard laboratory fermentation tanks, used in the Antibiotics Research Institute for studies in the technology of antibiotics, which have been slightly modified for continuous work. The tanks are made of stainless steel AKVN, the vessel itself being a cylinder with a total capacity of 20 l. (working capacity 5–15 l.). These tanks have been described in detail elsewhere.[31]

Sterilization Tank

For sterilization and preparation of the medium a stainless steel fermentation tank of 90 l. working capacity was used. The contents of the tank are agitated by a paddle impeller on a shaft. The shaft is driven by a 1·5 h.p. motor by means of a belt pulley. The tank is further equipped with a ring sparger and a baffle, and equipment for sampling and for injection of auxiliary substances. Medium is sterilized and cooled by means of coils with either steam or cold water. Air entering the tank through the ring sparger is sterilized by a filter packed with glass wool. On the lower end of the tank there is the closing valve, connected with the reservoir by means of piping.

Reservoir

The reservoir is an ordinary laboratory fermentation tank, the only difference being that the air entering the tank is not dispersed by means of the ring sparger but merely introduced above the surface of the medium. In this way foaming of the medium is prevented and the useful capacity of the tank is increased.

Electromagnetic Solenoid Valve

For the regulation of the injections of medium into the tank and for the outlet of fermented medium from the tank an electromagnetic solenoid valve with nominal inside diameter of 4 mm and nominal pressure of 6 atm, made of stainless steel, was used. It functions in such a way that with connection of current the coil attracts the core which simultaneously lifts the needle closing the upper part of the chamber so that medium is allowed to flow through the valve. Upon interruption of current the core falls by gravity into the lower part of the chamber taking with it the needle which stops the flow of medium. The valve is connected with the piping by means of a cone with a cap nut.

Relay Switch

The solenoid valves are governed by an automatic regulator. Various parts of the automatic regulator and their connection are

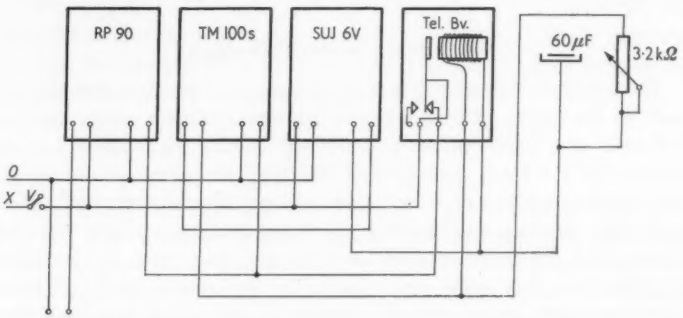

Fig. 2. Schematic sketch of the automatic regulator of injections

V—net switch
O—neutral wire
X—current
TM 100s time relay
SUJ 6V rectifier
Tel. Bv. telephone relay
RP 90 relay connecting the circuit of the valves V_1–V_4 of Fig. 1.

shown schematically in Fig. 2. It functions as follows: by connecting the net switch V, the time relay (TM 100s), adjustable from 2 to 100 sec, is brought into action. This relay determines the 'closed' period between two injections. After this period, the relay connects the d.c. 6 V circuit feeding the coil of the telephone relay (Tel. Bv.), and the electrolytic condenser (60 μF) with a parallel wire potentiometer of 3·2 kΩ by which the duration of the injection is determined. This potentiometer dissipates, more or less according to its position, the charge on the condenser feeding the coil of the telephone relay. By attraction of the core of the relay Tel. Bv. the feeding circuit of the timing relay TM 100s is instantly interrupted, whereas the condenser continues to feed the coil of the relay Tel. Bv. until discharge. After that the core of the telephone relay falls again connecting the circuit of relay TM 100s determining again the interval between the injections. The relay RP 90 connects the circuit of the valves.

Maintenance of Constant Level of Medium in Fermentation Tanks

A constant volume of medium in the fermentation tanks is maintained by an overflow pipe reaching to the level of the fermentation liquid. The amount of outflowing liquid is governed by intermittent make-and-break of the electromagnetic solenoid valve, the function of which is synchronized with that of the valve for the inlet of medium.

Description of the Procedure

The sterilization tank is sterilized by steam passing through the coils in the tank. The reservoir, the fermentation tanks and the collector are independently sterilized in the box autoclave for 4 h at 125 °C. Each tank is sterilized with the appropriate part of the piping and the closing valve without the electric coil, cover and cap. During sterilization all valves and taps of the tank are closed and free connections are wrapped in cotton wool. After sterilization the tanks are placed in the water bath. This is equipped with an automatic supply of cold water governed by a pneumatic registrating temperature regulator with a capillary thermometer as a sensing device and a pneumatic membrane valve. The reservoir (R) is connected with the sterilization

tank (S) by means of piping. The rest of the tanks (F_1–F_3) are so connected that the piping with the valve of one tank is connected with the corresponding part of the piping of the second tank by means of a cap nut. After the whole system has been so connected, the electric coils, covers and caps are fixed in place.

The medium is prepared and sterilized in the sterilization tank. After sterilization, medium from this tank is let into the other tanks except the collector. After consumption of all of the medium from the sterilization tank, this tank is used again for the preparation of new sterile medium. Medium in the tanks is inoculated with an inoculum from a flask by a pipette, supply and outlet of air is connected, and the aeration and over-pressure of air in the tank is regulated to the desired value.

Experimental Results

The equipment described above was used to study the possibility of continuous biosynthesis of streptomycin. *Streptomyces griseus*, strain LS–1, and medium of the following composition were used in the experiments: glucose 3·3 per cent, corn-steep solids 0·66 per cent, ammonium sulphate 0·9 per cent, monopotassium phosphate 0·02 per cent, calcium carbonate 0·6 per cent and ferrous sulphate 0·003 per cent.

The medium was aerated with 1·0 volume of air/min and stirred at 400 rev/min. The rate of oxygen transfer as measured by the sulphite oxidation was 2,500 ml O_2/l.h. The temperature during growth was maintained at 28°C and the tanks were inoculated with 100 ml of a 48 h inoculum from a flask.

Single-stage Process

At first, continuous cultivation in a single vessel was employed. Fig. 3 shows the patterns of pH and streptomycin concentration at a dilution rate of $D = 0{\cdot}02\ h^{-1}$. After starting the flow of the nutrient medium, the quantity of antibiotic constantly decreased and steadied at about 200 units of streptomycin/ml; when the flow of the nutrient medium was stopped, the quantity of antibiotic increased again until all glucose was utilized. Maintenance of a constant production of streptomycin between the

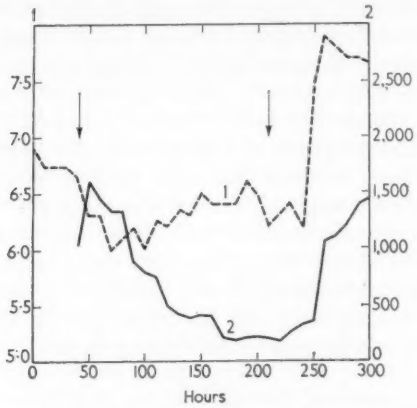

Fig. 3. Continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}02\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml). The arrows indicate the start and the end of the flow of the nutrient medium

limits of 2,000–2,500 u./ml was possible only with a very low dilution rate of $D = 0{\cdot}01\ h^{-1}$ (Fig. 4).

The process of biosynthesis for most antibiotics may be divided into at least two phases—a growth phase and a production phase.[32] The main synthesis of antibiotic does not begin until

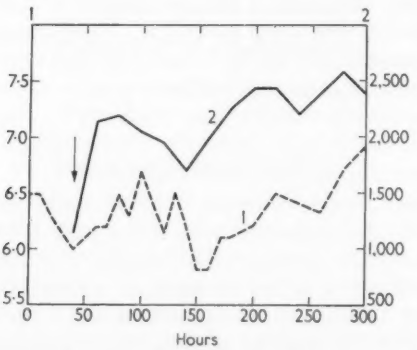

Fig. 4. Continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}011\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml). The arrow indicates the start of the flow of the nutrient medium

the growth of culture has largely stopped (Fig. 5). This is a typical example of a process in which the formation of the product is not connected with the growth of the micro-organism. The conversion of such a process into a one-stage, continuous process is therefore very difficult and may even be impossible because the dilution rate optimal for the growth of the mycelium is far greater than that for biosynthesis of antibiotic. We therefore considered the possibility of dividing the whole fermentation into several stages, with a three-stage process proving the most advantageous.

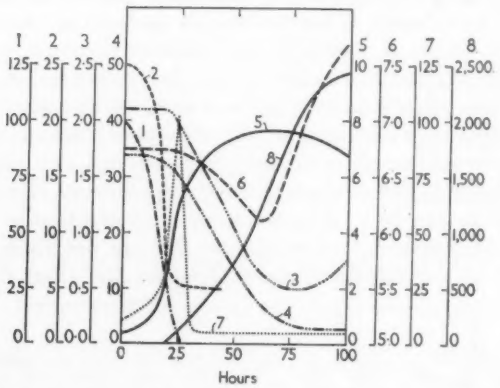

Fig. 5. Metabolic changes in the submerged culture of *Streptomyces griseus*. (1) concentration of phosphorus (μg/ml); (2) concentration of α-amino-nitrogen (mg/10 ml); (3) concentration of ammonia nitrogen (mg/ml); (4) concentration of glucose (mg/ml); (5) dry wt mycelium (mg/ml); (6) pH; (7) concentration of pyruvate (μg Na pyruvate/ml); (8) concentration of streptomycin (u./ml)

Multi-stage Operation

According to the course of metabolic changes determined in batch fermentation (Fig. 5) it is possible to divide the development of the culture of *Streptomyces griseus* into these three stages:

(1) Logarithmic growth of the mycelium accompanied by a rapid consumption of amino-nitrogen and phosphorus in the medium and by a steep increase and subsequent steep decrease of the level of keto-acids in the medium.

(2) Rapid consumption of reducing substances and ammonia

nitrogen, a decrease in pH and increasing formation of the antibiotic.

(3) Rapid synthesis of the antibiotic accompanied by a rapid increase in pH.

The operating process was divided into three steps corresponding to the above three stages; in the first stage continuous multiplication of the mycelium, in the second intense consumption of reducing substances and in the third main synthesis of the antibiotic, take place.

The most difficult problem was the determination of the velocity of exchange of one volume of medium in the first stage. From the curve of mycelial dry weight with time, determined for batch fermentations, the weight increase per hour between 10–60 h was calculated by a method described previously.[33] The shortest period of exchange of one volume of medium with maintenance of equilibrium conditions corresponded to a dilution rate of $D = 0{\cdot}2\ h^{-1}$; in this case, however, the amount of mycelium in the medium was low. A satisfactory concentration of the mycelium in the nutrient medium (about 3·5 mg dry weight/ml) was achieved at the dilution rate of $D = 0{\cdot}1\ h^{-1}$ (Fig. 6). As the

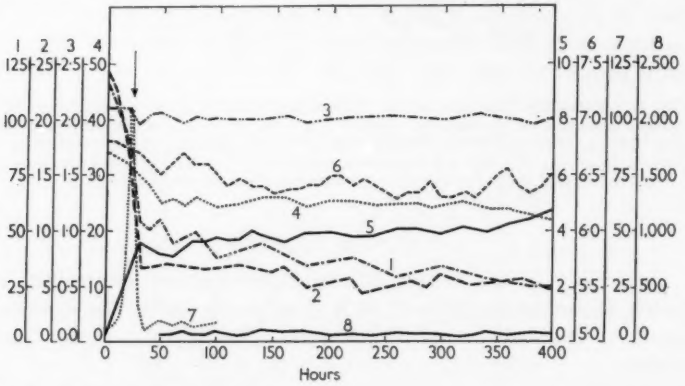

Fig. 6. Metabolic changes in the process of continuous cultivation of *Streptomyces griseus* in the first stage at the dilution rate $D = 0{\cdot}1\ h^{-1}$. (1) concentration of phosphorus (μg/ml); (2) concentration of α-amino-nitrogen (mg/10 ml); (3) concentration of ammonia nitrogen (mg/ml); (4) concentration of glucose (mg/ml); (5) dry wt mycelium (mg/ml); (6) pH; (7) concentration of pyruvate (μg Na pyruvate/ml); (8) concentration of streptomycin (u./ml). The arrow indicates the start of the flow of the nutrient medium

logarithmic growth of mycelium is accompanied by a parallel rapid increase of the level of keto-acids in the medium, flow was started when the maximum of keto-acids (about 100 μg/ml) was reached—usually about 23–24 h of cultivation.

Metabolic changes during the first, or rapid growth, stage of continuous fermentation are shown in Fig. 6. After the flow of the nutrient medium had been started (indicated by the arrow) a small decrease of the weight of mycelial dry matter set in and the weight did not stabilize at a constant value till after 50 h of cultivation. A similar decrease, occurring in the cultivation

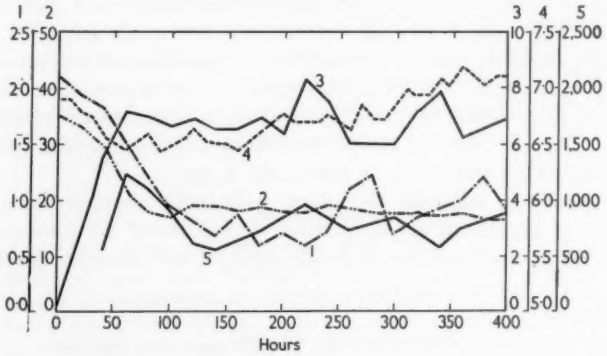

Fig. 7. Metabolic changes in the process of continuous cultivation of *Streptomyces griseus* in the second stage at the dilution rate $D = 0{\cdot}05\ h^{-1}$. (1) concentration of ammonia nitrogen (mg/ml); (2) concentration of glucose (mg/ml); (3) dry wt mycelium (mg/ml); (4) pH; (5) concentration of streptomycin (u./ml)

of bacteria, is ascribed by some workers to the distribution of generation times in the culture. It was found that the amount of ammonia nitrogen remained constant at the initial level during the whole process of fermentation while glucose and amino-nitrogen fell gradually. Investigations concerning the growth limiting factor were not successful. During the whole process of continuous fermentation it was possible to observe a small, gradual decrease in phosphorus and amino-nitrogen content and a slight increase in the weight of mycelial dry matter. This was most probably caused by the growth of micro-organisms on the walls of the fermenting vessel. These micro-organisms could be

washed down into the nutrient medium again, increasing the concentration of micro-organisms and causing an increased consumption of some of the nutrients. The amount of antibiotic in the medium varied from zero to 100 μ/ml in this first stage.

The process of the second stage is shown in Fig. 7. After 50 h of cultivation a strong growth of mycelium on the walls of the fermenting vessel was observed. The utilization of nutrients and the weight of mycelial dry matter varied considerably during the fermentation. Evidence was obtained that phosphorus and amino-nitrogen were entirely utilized so that they could be considered as factors limiting the growth. Adding both phosphorus and amino-nitrogen during fermentation did not, however, increase the weight of dry mycelium. Ammonia-nitrogen was partially utilized, but its content in the medium varied considerably in this stage. This may be explained by a partial autolysis of the hyphae which was also ascertained by microscopic observation. The content of streptomycin steadied after the initial increase within the limits of 500–1,000 u./ml.

It was very difficult to follow metabolic changes and the amount of mycelium in the medium during the third, final stage. The adherence of mycelium to the walls of the fermenting vessel

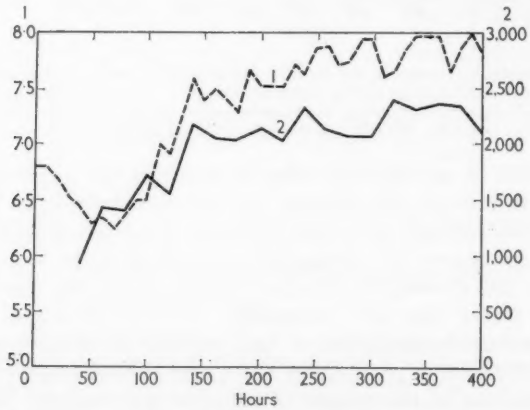

Fig. 8. Third stage continuous cultivation of *Streptomyces griseus* at the dilution rate $D = 0{\cdot}033\ h^{-1}$. (1) pH; (2) concentration of streptomycin (u./ml)

was very great and caused considerable differences in nutrient levels in the medium; therefore only the level of antibiotic and changes of pH were surveyed (Fig. 8). It is worth noting that even in this stage, at a low dilution rate, glucose and ammonia nitrogen were not entirely utilized and were removed unused. This factor, when applied to an industrial facility on a larger scale, would make this system less advantageous economically than the more common batch process.

In the whole equipment the flow velocity was so adjusted as to exchange the 5 l. of fermentation liquid in the first stage in 10 h. In the second stage a doubled residence time was achieved by doubling the volume of the liquid to 10 l. at equal flow velocity. In the third stage the volume was adjusted to 15 l. of medium for the same reason, giving a dilution rate of $D = 0{\cdot}033\ h^{-1}$.

The total fermentation operating time amounted to 300–400 h, with yields of 2,000–2,500 u. of streptomycin/ml, without any sign of degeneration or contamination of the culture.

References

[1] Novick, A. *Annu. Rev. Microbiol.*, 9 (1955), 97
[2] Monod, J. *Ann. Inst. Pasteur*, 79 (1950), 390
[3] Novick, A. and Szilard, L. *Science*, 112 (1950), 715
[4] Novick, A. and Szilard, L. *Proc. nat. Acad. Sci., Wash.*, 36 (1950), 708
[5] Northrop, J. H. *J. gen. Physiol.*, 38 (1954), 105
[6] Finn, R. K. and Wilson, R. E. *J. Agr. Food Chem.*, 2 (1954), 66
[7] Maxon, W. D. *Appl. Microbiol.*, 3 (1955), 110
[8] Herbert, D., Elsworth, R. and Telling, R. C. *J. gen. Microbiol.*, 14 (1956), 601
[9] Golle, H. A. *J. Agr. Food Chem.*, 1 (1953), 789
[10] Bryson, V. *Science*, 116 (1952), 48
[11] Anderson, P. A. *J. gen. Physiol.*, 36 (1953), 733
[12] Fox, M. S. and Szilard, L. *J. gen. Physiol.*, 39 (1955), 261
[13] Kubitschek, H. E. *J. Bact.*, 67 (1954), 254
[14] Haan de, D. G. and Winkler, K. C. *Leeuwenhoek ned. Tijdschr.*, 21 (1955), 33
[15] Perret, C. J. *J. gen. Microbiol.*, 16 (1957), 250
[16] Gerhardt, P. *J. Bact.*, 52 (1946), 283
[17] Formal, S. B., Baron, L. S. and Spilman, W. *J. Bact.*, 72 (1956), 168
[18] Novick, A. and Szilard, L. *Cold Spr. Harb. Symp. quant. Biol.*, 16 (1951), 337
[19] Rogers, H. J. *J. gen. Microbiol.*, 16 (1957), 22
[20] Cohn, M. and Torriani, A. M. *Biochim. biophys. Acta*, 10 (1953), 280

[21] Rogers, L. A. and Whittier, E. O. *J. Bact.*, 20 (1930), 127
[22] Beran, K. in *Continuous Cultivation of Microorganisms, A Symposium*, pp. 122–156 (1958), Prague; ČSAV
[23] Harris, E. E., Saeman, I. P., Marquardt, R. R., Hannan, M. J. and Rogers, S. C. *Industr. Engng. Chem.* (*Industr.*), 40 (1948), 1216
[24] Reiser, C. O. *J. Agr. Food Chem.*, 2 (1954), 70
[25] Bilford, H. R., Scalf, R. E., Stark, W. H. and Kolachov, P. J. *Industr. Engng. Chem.* (*Industr.*), 34 (1942), 1406
[26] Ruf, E. W., Stark, W. H., Smith, L. A. and Allen, E. E. *Industr. Engng. Chem.* (*Industr.*), 40 (1948), 1154
[27] Dyr, J., Protiva, J. and Praus, R. in *Continuous Cultivation of Microorganisms, A Symposium*, pp. 210–226 (1958), Prague; ČSAV
[28] Elsworth, R., Meakin, L. R. P., Pirt, S. and Capell, G. H. *J. appl. Bact.*, 19 (1956), 264
[29] Harrison, R. W. *J. appl. Chem., Lond.*, 8 (1958), 68
[30] Hedén, C.-G., Holme, T. and Malmgren, B. *Acta path. microbiol. scand.*, 37 (1955), 42
[31] Sikyta, B., Herold, M., Šlechta, J. and Zajíček, J. *Kvasný průmysl*, 4 (1958), 275
[32] Doskočil, J., Sikyta, B., Kašparová, J., Doskočilová, D. and Zajíček, J. *J. gen. Microbiol.*, 18 (1958), 302
[33] Adams, S. L. and Hungate, R. E. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 1815

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A Kinetic Study of the Lactic Acid Fermentation. Batch Process at Controlled pH*

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Summary. Kinetic data are needed to develop basic understanding of fermentation processes and to permit rational design of continuous fermentation processes.

The kinetics of the fermentation of glucose to lactic acid have been studied at six constant pH levels between 4·5 and 6·0 by measuring the instantaneous rates of bacterial growth and of lactic acid formation throughout each fermentation.

It was found that the instantaneous rate of acid formation dP/dt, could be related to the instantaneous rate of bacterial growth dN/dt, and to the bacterial density N, throughout a fermentation at a given pH, by the expression

$$\frac{dP}{dt} = \alpha \frac{dN}{dt} + \beta N$$

where the constants α and β are determined by the pH of the fermentation.

Introduction

Largely because of products such as antibiotics, vitamins, feed supplements, and blood plasma expanders, fermentation as an industrial method for making speciality chemicals is attracting wide attention. Consequently, a better understanding of the kinetics of fermentation is becoming increasingly important and, although the advantages and economies of continuous fermentation have been widely reported,[7, 10, 22, 29] instantaneous rate measurements which are needed to predict continuous operation from batch data are not available.[6, 7]

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Because its rate processes are relatively simple, the lactic acid fermentation, particularly one using a homofermentative thermophilic organism such as *Lactobacillus delbrueckii* is well suited for basic fermentation studies. Inasmuch as a single material (glucose) is converted to a single product (lactic acid) with negligible amounts of intermediate and side products, attention may be focused on three primary processes—rates of nutrient disappearance, product formation, and bacterial growth. Also, sterility demands are not high, pH is easily controlled, and high aeration is not needed. Moreover, there is a need for continued research into the lactic acid fermentation in view of the attractive potential of lactic acid as a versatile intermediate, especially in plastics.[4, 25, 27]

The data reported here show the changes in nutrient concentration, product concentration, and bacterial density which occur during fermentation at constant temperature and controlled pH levels. Graphical differentiation of these curves provides the instantaneous rate data leading to the kinetic relationships developed. The kinetic equations relate quantitatively the rates of acid production to the rates of bacterial growth and to the bacterial population. The constants of the equation are a function of the pH of the fermentation. The application of these rate data to the design of continuous fermentation processes have been reported.[21]

This study of the batch process represents a continuation of research on the lactic acid fermentation previously conducted in this laboratory,[2, 3, 16] in which the extreme influence of pH upon yields, degree of completion, and fermentation rate was established for lactic acid fermentations with the pH continuously controlled. The present work differs from the earlier studies in that here it is the *instantaneous* rate of acid formation which is measured and followed throughout the fermentation rather than obtaining an average rate for the whole fermentation[16] or for a part of it.[2, 3] Moreover, instantaneous rates of bacterial growth are also determined in the present study, but were not in the earlier work.

Longsworth[17] and Longsworth and MacInnes[18–20] had earlier noted that a *Lactobacillus acidophilus* fermentation proceeded far more rapidly when the pH was controlled than when it was not

controlled. They also established methods for measuring instantaneous rates. Gillies[9] measured the instantaneous rate of acid formation during a lactic acid fermentation at a controlled pH of 5·4 and found that the rate was in part a function of the nutrilite (corn-steep liquour) concentration.

Experimental Equipment and Procedures

The equipment and methods used by Kempe[16] and Finn[3] have been modified for these studies of instantaneous rates. The experimental apparatus, Figs. 1–3, consisted basically of a fermentor plus its attendant services, including temperature control, pH control, introduction of carbon dioxide, and means for measurement of bacterial growth and acid production. The

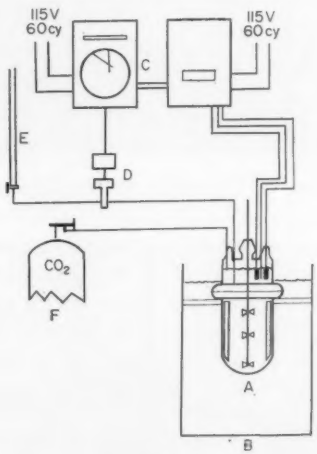

Fig. 1. Sketch of equipment for fermentation and pH control

A. Fermentor with stirrer, baffles, pH electrodes and ground, inlets for carbon dioxide and sodium carbonate solution
B. Constant temperature water bath (45 °C)
C. pH meter and pH Recorder–Controller
D. Pump for carbonate solution
E. Measuring reservoir (burette) for carbonate solution
F. Carbon dioxide tank

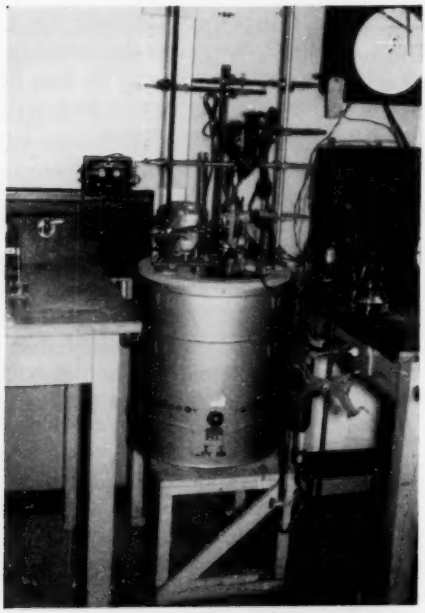

Fig. 2. General view of equipment for rate studies in batch fermentation

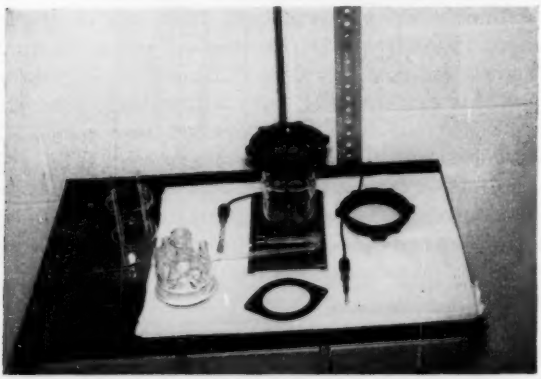

Fig. 3. Dismantled fermentor with agitator and pH electrodes

two-litre fermentor, also designed for continuous fermentation studies, was a two-piece, borosilicate-glass reaction flask fitted with baffles and stirrer. Of the six top openings of standard taper ground glass grouped about the central agitator entry of the fermentor, three were used for the pH meter electrodes and ground, and the remaining three were used for sampling, for introduction of alkali, and for introduction of carbon dioxide.

A Beckman Model RX pH meter and Bristol Pyromaster Potentiometer continuously recorded the pH and controlled it to any desired level between pH 3·0 and pH 10·0 by the on–off operation of a small proportioning pump, which injected a 2·5 N solution of sodium carbonate into the fermentation mash as required. Control was within $\pm 0{\cdot}05$ pH units. The equipment was grounded to the soil to prevent erratic operation of the meters.[3, 16] The pH electrodes were equipped with standard taper ground glass sleeves to fit into the top openings of the fermentor.

All fermentations were conducted at 45°C using the homofermentative *L. delbrueckii* NRRL–B445 obtained originally from the United States Department of Agriculture for earlier studies.[3, 16] The species has been described elsewhere,[1] as have this strain and the culture techniques.[2]

In order to help exclude atmospheric oxygen, the surface of the medium was blanketed with carbon dioxide by slowly bleeding the gas into the reactor during the fermentations.[2, 3] Since the fermentor was closed, no evaporation losses occurred.

The basal medium was an aqueous solution containing 5 per cent anhydrous dextrose, 3 per cent dehydrated yeast extract, and added mineral salts.[3] The yeast extract–glucose solution and the fermentor assembly were essentially sterilized by bubbling steam into the medium in the fermentor for two hours while holding it at 100°C. In order to prevent the formation of a troublesome flocculent precipitate, the mineral salts solutions were sterilized separately and added to the cooled medium just before inoculation. The volume was then adjusted with sterile water.

After calibration against buffer solutions, the pH electrodes were sterilized by immersing them in a dilute quaternary ammonium solution and rinsing them twice in sterile water before inserting them into the fermentor.

Prior to inoculation, the optical density of the 18 to 24-h starter culture was measured, and the volume of the inoculum was chosen such that it contained 180 units of optical density (to be defined below). The volume of inoculum was usually between 50 and 80 ml for an initial charge of 1600 ml.

During each fermentation the calibration of the pH recorder-controller was checked several times by measuring the pH of samples of the fermentation broth on a second meter. Once the fermentation had started, no special precautions were taken to maintain sterile conditions since the high temperature and low pH of the medium, and the heavy inoculum and rapid rate of fermentation, all tended to discourage the growth of chance contaminants. There was no evidence of contamination in any of the batch fermentations conducted during this study.

Measurements

For the rate studies the two processes, bacterial growth and lactic acid synthesis, were closely measured during the entire fermentation.

The lactic acid was determined as it was formed by measuring the addition to the fermentation mash of an alkali solution (2·5 N sodium carbonate with 5 g hexametaphosphate added per litre to compensate for depletion of phosphate during the fermentation). The alkali was pumped from a burette and the volume of alkali was noted at frequent intervals, preferably at the instant the pump was activated. Each millilitre of alkali is theoretically equivalent to 0·2252 g of lactic acid, but, because of the low pH levels, it was necessary to correct for the undissociated acid. The validity of measuring the lactic acid formed by measuring the volume of carbonate added, was confirmed by analyzing the final fermentation broth specifically for lactic acid.

Since the growth measuring techniques used in this study are somewhat novel, the reasons for their selection and the actual procedures used are described.

For this study indirect schemes such as measuring carbon dioxide production, nutrient consumption or other results of growth were unsuitable for measuring the bacterial growth rates since they do not necessarily remain proportional to growth.

Cumbersome direct weighing schemes were also excluded because they do not permit making the frequent measurements necessary for rate studies.

For measuring bacterial growth, optical density, which measures bacterial density, is preferred to plate counting and direct counting methods which measure cell concentration.[11, 13, 23] Cell concentration is defined[23] as the number of cells per unit volume of solution, while bacterial density is defined as the dry weight of bacteria per unit volume of solution. Because the average size of the bacterial cells changes during fermentation, bacterial density is more closely related to the quantity of bacterial protoplasm, and therefore, more closely related to enzyme quantity.

Optical density techniques are preferred for measuring bacterial density[11, 23] since they are the most convenient, and because the optical density can be expected to remain proportional to the bacterial density during the positive phases of growth cycle.

Since correlations of optical densities with plate counts were erratic, primarily because the plate counts failed to distinguish between single cells and groups or chains of cells, all results are reported in terms of optical densities.

Optical densities for this study were measured on an Industrial Model LCZ Leitz Rouy-Photrometer, using the 610 filter (maximum transmission at 610μ) with a 10 mm $\times$ 10 mm absorption cell of 9 ml capacity furnished by the manufacturer. The instrument dial reads in per cent transmission, which may be converted to optical density by the equation

$$D = \log_{10} I_0 - \log_{10} I$$

where D is the optical density, I is the instrument reading of the sample in per cent transmission, and I_0 is the instrument reading for the blank in per cent transmission. The instrument was adjusted to $I_0 = 100$ for a distilled water blank, and the correction was made later for the optical density of the sterile medium.

For convenience, and in order to represent the optical densities as the concentrations which they actually are, bacterial densities are reported as 'units of optical density per millilitre' or U.O.D./ml, which is defined as follows: the bacterial suspension is diluted with distilled water until the dilution reads 75 per cent transmission on the Leitz Rouy-Photrometer (75 per cent transmission is

equal to an optical density of 0·125); if r is the number of times which the sample is diluted, the bacterial density of the original sample in U.O.D./ml is given as $N = 0{\cdot}125r$.

In order that all samples would not have to be diluted to exactly 75 per cent transmission, a chart was prepared which converted a per cent transmission reading of the photrometer directly to an optical density value corrected for experimental deviations from the Beer–Lambert Law. For the *L. delbrueckii* used in this study, one unit of optical density corresponded to between 150,000,000 and 450,000,000 cells as determined by plate counts.

Instantaneous rates of growth and of acid formation were determined by graphical differentiation of curves of values computed from the original measurements of alkali addition and of optical density versus time after inoculation. The specific rate of growth and the specific rate of acid formation were determined by dividing the instantaneous rates by N, the bacterial density at that time. Included in the calculations are procedures, originally outlined by Longsworth and MacInnes,[19] to correct for the continual dilution of the medium by alkali solution, and for the withdrawal of samples. Errors from each of these sources, if neglected, can be as high as 15–20 per cent.

For each run a sample of the final fermentation broth was frozen and analyzed later for glucose, using the Hodge and Davis modification of the Somogyi semimicro method for reducing sugars.[15] The Friedemann–Graeser method[5] was used to analyze for lactic acid. Results of the lactic analyses were erratic, however, until two modifications recommended by Smith[28] were incorporated into the analysis: specifically, that the time of the Van Slyke pretreatment was limited to 1 min, and that a 0·01 N solution of potassium permanganate was used as the oxidizing agent in place of colloidal manganese dioxide. With these two modifications, recoveries of lactic acid ran consistently above 96 per cent and often at about 99 per cent for prepared samples.

Discussion

The experimental data and the curves presented here are derived from six fermentations, each of which was continuously controlled at a different pH level (6·0, 5·6, 5·4, 5·2, 4·8 and 4·5).

All fermentations were conducted at the same temperature (45°C), with the same organism (*L. delbrueckii* NRRL–B445), and on the same medium (an aqueous solution of 5 per cent glucose fortified with 3 per cent yeast extract and additional mineral salts). As an example, the rate information for the fermentation at pH 6·0 is tabulated in Tables I and II, and the curves for the fermentations at pH 6·0, 5·4 and 4·5 are shown in Figs. 4–7.

The two important processes, growth and acid formation, are shown in three different ways in Figs. 4–6. Firstly, both are

Table I. Bacterial density during batch fermentation*

Time, h	*N*, U.O.D./ml	Time, h	*N*, U.O.D./ml
0·10	0·11	8·30	3·10
1·00	0·12	8·80	3·97
1·50	0·14	9·30	4·94
2·00	0·17	9·35	5·02
3·20	0·30	9·55	5·53
3·70	0·39	9·80	5·99
4·20	0·49	9·85	6·10
4·70	0·65	10·10	6·49
4·95	0·73	10·35	6·86
5·20	0·80	10·55	7·05
5·45	0·89	10·80	7·35
5·50	0·90	11·05	7·70
5·70	1·01	11·10	7·73
5·75	1·01	11·30	8·01
5·90	1·11	11·35	8·05
6·00	1·13	11·80	8·39
6·20	1·25	12·10	8·62
6·50	1·37	12·35	8·75
6·75	1·56	12·85	9·03
7·00	1·76	13·60	9·47
7·25	1·93		
7·50	2·22		
7·75	2·48		
8·00	2·79		
8·25	2·95		

* At 45°C, pH controlled at 6·0, on a 5 per cent glucose medium fortified with 3 per cent yeast extract and added mineral salts, *L. delbrueckii*.

Table II. Growth and lactic acid synthesis during batch fermentation†

Time, h	N* U.O.D./ml	P mg/ml	$\frac{dN}{dt}$ U.O.D./ml h	$\frac{dP}{dt}$ mg/ml h	k h^{-1}	$\frac{1}{N}\frac{dP}{dt}$ mg/U.O.D. h
1·00	0·12		0·04		0·34	
1·50	0·14		0·05			
2·00	0·17		0·06		0·36	
2·50	0·22	0·8	0·09	0·4	0·40	1·8
3·00	0·28		0·13		0·46	
3·50	0·35	1·25	0·16	0·56	0·47	1·60
4·00	0·45	1·6	0·21	0·74	0·478	1·66
4·50	0·57	1·95	0·27	0·92	0·48	1·61
5·00	0·72	2·5	0·35	1·20	0·48	1·66
5·50	0·91	3·2	0·44	1·51	0·48	1·66
6·00	1·15	4·0	0·55	1·81	0·48	1·58
6·50	1·39	5·0	0·67	2·10	0·48	1·51
7·00	1·76	6·1	0·85	2·54	0·48	1·45
7·50	2·21	7·6	1·06	3·48	0·478	1·58
8·00	2·79	9·5	1·33	4·37	0·475	1·57
8·50	3·46	12·0	1·63	5·58	0·470	1·62
9·00	4·31	14·9	1·99	6·53	0·460	1·51
9·50	5·35	18·2	2·09	7·05	0·390	1·32
10·00	6·30	21·6	1·70	7·34	0·270	1·16
10·50	7·00	25·1	1·30	7·19	0·186	1·03
11·00	7·60	28·6	1·06	7·05	0·140	0·93
11·50	8·14	31·8	0·89	6·44	0·109	0·79
12·00	8·51	34·9	0·74	6·24	0·087	0·72
12·50	8·84	37·9	0·62	5·86	0·070	0·66
13·00	9·13	40·7	0·54	5·57	0·059	0·61
13·50	9·40	43·3	0·44	5·00	0·047	0·53
14·00		44·9				

* Interpolated.

† At 45°C, pH controlled at 6·0, on a 5 per cent glucose medium fortified with 3 per cent yeast extract and added mineral salts, *L. delbrueckii*.

plotted against fermentation time in Fig. 4 with the bacterial density N, expressed as units of optical density per millilitre, and the product or lactic acid concentration P, expressed as milligrams of acid per millilitre of solution. Bacterial density curves are also presented in the semi-logarithmic plots of Fig. 7, where growth at the several pH levels is compared. Secondly,

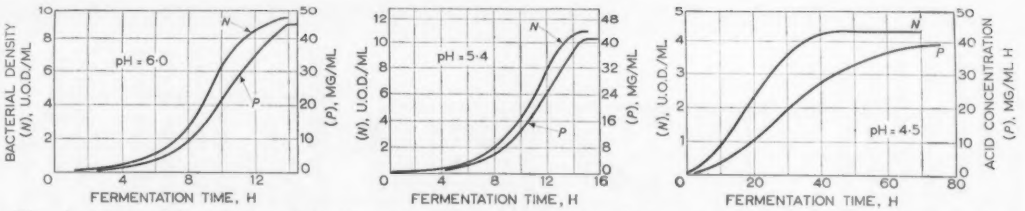

Fig. 4. Bacterial growth and lactic acid synthesis during batch fermentations at controlled pH levels

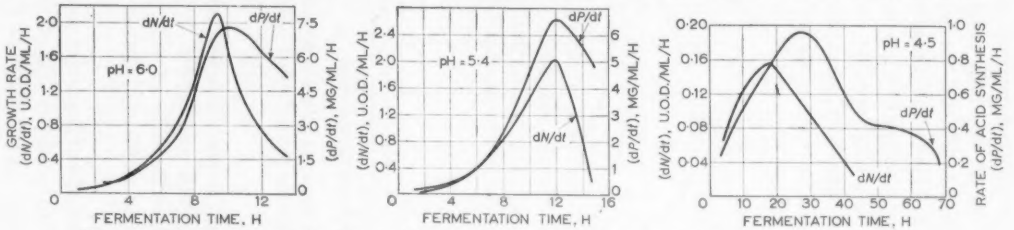

Fig. 5. Bacterial growth rate and lactic acid synthesis rate versus time during batch fermentations at controlled pH levels

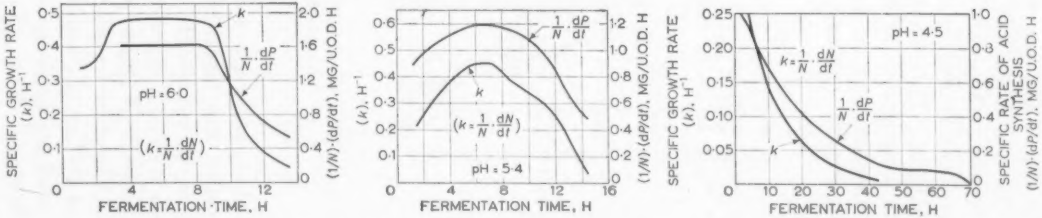

Fig. 6. Specific rate of bacterial growth and of lactic acid synthesis versus time during batch fermentations at controlled pH levels

in Fig. 5 the two rates are plotted against fermentation time. The rate of growth is shown as the rate of change of bacterial density with respect to time (dN/dt), and the rate of acid synthesis is shown as the rate of change of lactic acid concentration with time (dP/dt). These curves were obtained by graphical differentiation of plots similar to Fig. 4. Finally, specific rates, defined as the rates per unit of bacterial quantity, are plotted against time in Fig. 6. Specific growth rate is computed as $(1/N)(dN/dt)$,

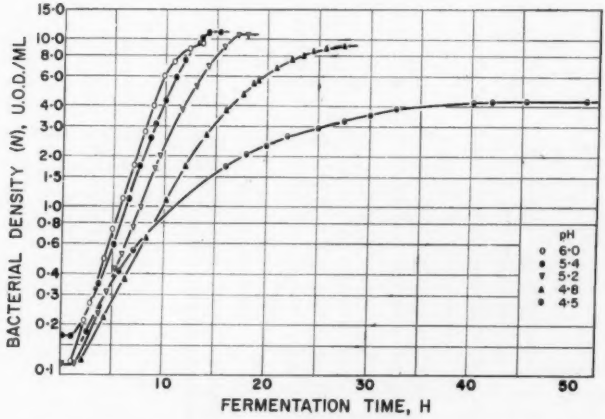

Fig. 7. Semi-logarithmic plot showing increase of bacterial density during batch fermentations at controlled pH levels

whose units are reciprocal hours. Specific growth rate is also called the logarithmic growth coefficient k, or the Naperian growth rate.[23, 24] The specific rate of acid synthesis is given as $(1/N)(dP/dt)$ and has the units milligrams of acid per hour per unit of optical density.

The growth rates as functions of bacterial density are compared at the six pH levels in Fig. 8, and in Fig. 9 the specific growth rates are similarly compared. Plots such as Figs. 8 and 9 where bacterial density is the parameter are most useful in detecting trends, and are also important in continuous fermentation studies.[21]

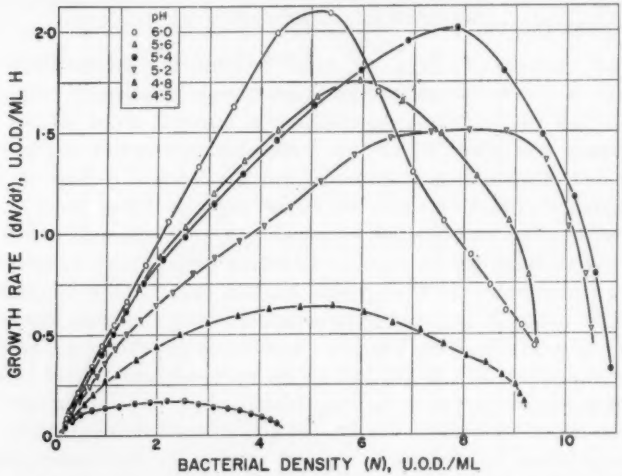

Fig. 8. Bacterial growth rates as functions of the bacterial density during batch fermentations at controlled pH levels

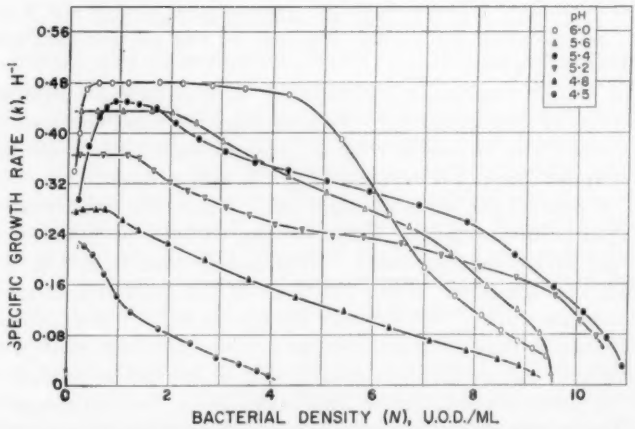

Fig. 9. Specific bacterial growth rates as functions of the bacterial density during batch fermentations at controlled pH levels

Growth as a Function of pH

The curves of Figs. 4 and 7 confirm earlier observations[3, 18, 19, 26, 27] that fermentation time is lengthened markedly when the pH of the fermentation is lowered from 6·0 to 4·5. However, the effect of pH on fermentation kinetics is complex, and new information is gained from the curves of Figs. 4–9 by studying its effect on some individual aspects of growth.

For example, as the pH at which a fermentation is controlled is lowered from 6·0 to 4·5, the following effects may be observed from the curves: (1) the specific growth rate for the logarithmic growth phase k, i.e., the logarithmic growth constant, decreases markedly, (2) the length of the logarithmic growth phase steadily decreases from 8 h at pH 6·0 to an unmeasurably short time at pH 4·5, (3) furthermore, the logarithmic phase ends at successively lower bacterial density levels, (4) the overall fermentation time is lengthened, (5) the fermentation attains its maximum growth rate (dN/dt), successively later in the growth cycle, (6) the greatest bacterial density is attained by fermentation at pH 5·4, and (7) a change in the shapes of the curves for growth rate, for specific growth rate, and for rate of acid synthesis also occurs at pH 5·4.

In addition to the above changes at pH 5·4, it might also be noted here that this same pH 5·4 appears later in this study as a point of inflection for α (Fig. 10), and as the pH for maximum yield for lactic acid.

For batch fermentations there is little practical difference between pH 5·4 and pH 6·0. In both cases the total fermentation times are about the same (Fig. 7), approximately the same level of bacterial density is reached (Fig. 7), and conversion to lactic acid is nearly the same at each pH. From the standpoint of continuous fermentation, however, the differences between the two pH levels are significant. This can be seen from Fig. 8 by noting the difference in the positions of the growth rate curves at these two pH's. To explain briefly, at pH 6·0 a continuous fermentation may be conducted at a very high flow rate (which means that the nominal holding time is short) but at a relatively low bacterial density, below 5·0 U.O.D./ml. This is because the maximum growth rate occurs at a lower bacterial density than with pH 5·4. At this lower pH, however, one can operate a

continuous fermentation at a higher bacterial density for a given flow rate than would be possible at pH 6·0. The differences are discussed more fully elsewhere.[21]

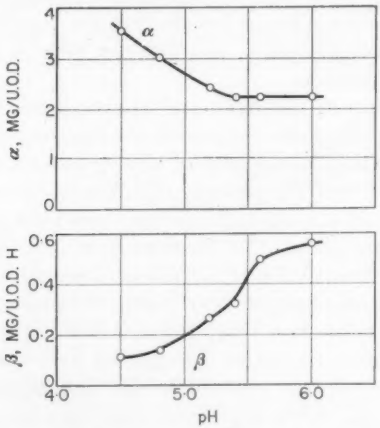

Fig. 10. The effect of pH on the coefficients α and β in the equation $(1/N)(dP/dt) = \alpha k + \beta$

Relation Between Acid Synthesis and Growth

During the fermentation, lactic acid production appears to parallel the growth of the organisms (Fig. 4). Like the growth rate, the rate of acid production rises to a maximum value in the latter part of each fermentation and then declines sharply from it (Fig. 5). The sharpness and short duration of these maxima, and the shortness of any other periods where the rate is approximately constant, demonstrate that the 'phase of constant rate of acid production' reported earlier[3] is a period of vigorous acid production where the 'constant rate' is in reality the average of rates that vary widely over the entire period.

The specific rates, both of growth and of acid production, are in a sense measures of the metabolic activity of the individual cells. It is to be expected, therefore, that if the lag phases are ignored, the specific rates are high in the early part of the fermentations, and that they decline steadily as the fermentation

proceeds due to the disappearance of nutrients and the accumulation of toxic products.[14] The curves of Fig. 6 do follow this pattern.

It is desirable, both for the subsequent studies of continuous fermentation and for a better insight into the batch fermentation, to establish the relationship between the rate of acid synthesis and the rate of growth.

An examination of the data and curves bears out neither of the two assumptions commonly made concerning this relationship. If, as is often assumed, the rate of acid synthesis is proportional to the number of bacteria present, then the bacteria should maintain the same level of metabolic activity and $(1/N)(dP/dt)$ should be constant throughout the fermentation. It is immediately clear from Fig. 6 that $(1/N)(dP/dt)$ does not remain constant in any of the fermentations except briefly during the phase of logarithmic growth, and that this first assumption is therefore not valid here. The second assumption, that the rate of acid synthesis is proportional to the rate of growth, is also inadequate as may be seen from Fig. 5. After attaining their maxima, the two rate curves diverge so widely that a simple proportionality obviously does not express the relationship between the two.

A reasonably close correlation can be obtained if it be assumed that the rate of acid synthesis is related *both* to the rate of growth and to the quantity of bacteria present, using the simplest possible relationship,

$$\frac{dP}{dt} = \alpha \frac{dN}{dt} + \beta N \tag{2}$$

where α and β are constants of proportionality.

It is easier to verify this assumed relationship and to evaluate the constants if the equation be modified by dividing by N to give

$$\frac{1}{N}\frac{dP}{dt} = \frac{\alpha}{N}\frac{dN}{dt} + \beta$$

Since, by definition, $k = (1/N)(dN/dt)$, the equation finally simplifies to

$$\frac{1}{N}\frac{dP}{dt} = \alpha k + \beta \tag{3}$$

When the experimental values of $(1/N)(dP/dt)$ are plotted against k in Fig. 11, the points fall close to a straight line, thus tending to confirm the validity of equations 3 and 2. Thus the rate of lactic acid synthesis is not a function of bacterial density alone, nor of growth rate alone, but it is a function of the bacterial density and of the rate of bacterial growth together.

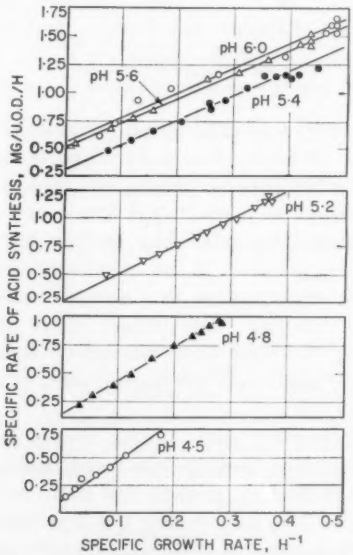

Fig. 11. Specific rate of lactic acid synthesis as a function of the specific rate of bacterial growth during batch fermentations at controlled pH levels

The constants α and β are determined from the plots of Fig. 11 where α is equal to the slope of the straight line and β is equal to the $(1/N)(dP/dt)$ intercept. The manner in which α and β vary with pH can be seen from Table III and from Fig. 10.

Equations 2 and 3 suggest that the production of lactic acid is related to two life processes. One can speculate that the cell dissimilates glucose to lactic acid in order to obtain the energy required to form new bacterial protoplasm, and at the same time it does so as a normal metabolic activity irrespective of

Table III. Experimental constants α and β of equations 3 and 2 for each pH level

pH	α	β
6·0	2·2	0·55
5·6	2·2	0·49
5·4	2·2	0·32
5·2	2·45	0·26
4·8	3·0	0·14
4·5	3·55	0·11

α has the units mg lactic/U.O.D.
β has the units mg lactic/U.O.D./h

growth. The rate of acid production per cell for the first process is represented by αk and is therefore proportional to the specific growth rate. For the second process the rate of acid production per cell is a constant at a given pH level. In the early phases of a normal fermentation when the specific growth rate is high, the first term of equation 3 is the important one, while towards the end of the fermentation the second term becomes more important. For 'resting' cells where there is supposedly no growth occurring, the first term should be zero and all acid is produced in accordance with the second term of the equation, the constant β.

It is also noteworthy that during the phase of logarithmic growth the two common assumptions mentioned above for relating rate of acid production to growth are both valid. This is true because $(dN/dt) = kN$, and during the logarithmic phase k is a constant, k_c. Thus one can state with equal validity that during the logarithmic phase the rate of acid production is proportional to the growth rate of the bacteria, or that during this phase the rate of acid production is proportional to the quantity of bacteria present. Neither statement was found to hold true outside of the period of logarithmic growth, while equation 3, of course, was found to apply throughout the entire fermentation cycle.

Nomenclature

D Optical density of a diluted sample

I Leitz Rouy-Photrometer instrument reading of diluted sample, per cent transmission

I_0	Instrument reading of blank, per cent transmission
k	Specific (Naperian) growth rate $\left(k = \frac{1}{N}\frac{dN}{dt}\right)$, h^{-1}
k_c	Logarithmic growth constant (constant value of k during phase of logarithmic growth), h^{-1}
$\log_{10}$	Logarithm to the base 10
N	Bacterial density, units of optical density (U.O.D.) per ml
P	Lactic acid or product concentration, mg/ml
r	Volume ratio of diluted sample to original sample for optical density measurements
$\frac{dN}{dt}$	Rate of bacterial growth, U.O.D./ml h
$\frac{dP}{dt}$	Rate of lactic acid synthesis, mg/ml h
$\frac{1}{N}\frac{dP}{dt}$	Specific rate of lactic acid synthesis, mg/U.O.D. h
α, β	Constants

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References

[1] Bergey, David H. *Bergey's Manual of Determinative Bacteriology*, 6th ed., p. 359 (1948). Baltimore: Williams and Wilkins Co.

[2] Finn, R. K. Ph.D. Thesis, Dept. of Chemical Engineering, University of Minnesota (1949)

[3] Finn, R. K., Halvorson, H. O., and Piret, E. L. *Industr. Engng. Chem. (Industr.)*, 42 (1950), 1857

[4] Fisher, C. H., and Filachione, E. M. U.S. Dept. of Agriculture, Bur. Agric. Industr. Chem., AIC–178 (1948); see also AIC–279 (1950)

[5] Friedemann, T. E. and Graeser, J. B. *J. biol. Chem.*, 145 (1932), 131

[6] Gaden, E. L., Jr. *Chem. & Ind. (Rev.)* (1955), 154

[7] Gaden, E. L., Jr. *Chem. Engng. Progr.*, 51 (1955), 540

[8] Gaden, E. L., Jr. *Chem. Engng.*, 63, No. 4 (1956), 159

[9] Gillies, R. A. Ph.D. Thesis, Dept. of Bacteriology, University of Michigan (1955)

[10] Golle, H. A. *J. Agr. Food Chem.*, 1 (1953), 789
[11] Gunsalus, I. C. *Bacterial Physiology*, Chap. IV. (1951). Ed., Werkman, C. H. and Wilson, P. W. New York: Academic Press.
[12] Hamer, W. J. and Acree, S. F. *J. Res. nat. Bur. Stand.*, 32 (1944), 215
[13] Henrici, A. T. *J. infect. Dis.*, 38 (1926), 54
[14] Hinshelwood, C. N. *The Chemical Kinetics of the Bacterial Cell.* (1946). London: Oxford University Press
[15] Hodge, J. E. and Davis, H. A. U.S. Dept. of Agriculture, Bur. Agric. Industr. Chem., AIC–333 (1952)
[16] Kempe, L. L., Halvorson, H. O. and Piret, E. L. *Industr. Engng. Chem. (Industr.)*, 42 (1950), 1852
[17] Longsworth, L. G. *J. Bact.*, 32 (1936), 307
[18] Longsworth, L. G. and MacInnes, D. A. *J. Bact.*, 29 (1935), 595
[19] Longsworth, L. G. and MacInnes, D. A. *J. Bact.*, 31 (1936), 287
[20] Longsworth, L. G. and MacInnes, D. A. *J. Bact.*, 32 (1936), 567
[21] Luedeking, R. and Piret, E. L. This Journal, 1 (1959), 431
[22] Maxon, W. D. *Appl. Microbiol.*, 3 (1955), 110
[23] Monod, J. *Annu. Rev. Microbiol.*, 3 (1949), 371
[24] Monod, J. *Ann. Inst. Pasteur*, 79 (1950), 390
[25] Needle, H. C. and Aries, R. S. *Sugar*, 44, No. 12 (1949), 32
[26] Porter, J. R. *Bacterial Chemistry and Physiology.* (1946). New York: John Wiley and Sons, Inc.
[27] Schopmeyer, H. H. *Industrial Fermentations*, Vol. 1, Chap. XII. Ed., Underkofler, L. A. and Hickey, R. J. (1954). New York: Chemical Publishing Co., Inc.
[28] Smith, R. J. *Analyt. Chem.*, 25 (1953), 505
[29] Underkofler, L. A. and Hickey, R. J., ed. *Industrial Fermentations*, Vol. 1. (1954). New York: Chemical Publishing Co., Inc.
[30] Wright, B. E. *J. Bact.*, 66 (1953), 407

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Fermentation Process Kinetics*

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Summary. Information on fermentation process kinetics is potentially valuable for the improvement of batch process performance; it is essential for continuous process design. An empirical examination of rate patterns in various fermentations discloses three basic types: (1) 'growth associated' products arising directly from the energy metabolism of carbohydrates supplied, (2) indirect products of carbohydrate metabolism and (3) products apparently unrelated to carbohydrate oxidation. Effects of operating variables on the primary kinetic processes, growth, sugar utilization and antibiotic formation, in the penicillin process, illustrate the special nature of this type.

Introduction

In the design of any chemical, or biochemical, process one must consider two more or less distinct aspects. First, there are the chemical reactions themselves and secondly, the numerous physical processes which precede, accompany and follow them. Some of these physical processes are quite clearly separate, like the purification of raw materials and products. Others, like the transport of materials to and from the surface of a solid catalyst, are intimately bound up with the reactions themselves.

For a long time, methods available for dealing with the physical aspects of chemical processes were better developed than those for handling the chemical changes themselves. This was largely the result of empirical simplifications offered by the 'unit operations' concept in chemical engineering. With the rapid development of chemical kinetics and, equally important, methods for applying kinetic relationships to process design, this disparity has been overcome.

* Presented at the 134th National Meeting of the American Chemical Society, Chicago, September 1958.

Kinetics is concerned with reaction rates in general; 'process kinetics' simply suggests a primary concern with the rates of commercially practised reactions and, particularly, with the effects of process variables on them.

Since fermentation is only another type of chemical process, albeit a special and complex one, possibilities for applying ideas and techniques developed for more conventional chemical systems should always be sought. This is especially true for kinetics. Although the study of fermentation rates is relatively new, it promises much for the fuller and more efficient exploitation of biochemical reaction systems.

Development of Fermentation Kinetics

Final product yields and substrate conversions were the only criteria of performance in early commercial fermentations. As the technology developed, however, greater attention was paid to time factors; 'productivity', the average rate of product formation (Fig. 1), soon became popular as a basis for comparison. On the other hand, instantaneous rates were largely ignored until the studies of gluconic acid production by Wells, Moyer, Gastrock *et al.*[12, 19, 20, 26] in the late 1930's. They were among the first to report rates of sugar utilization and acid formation in detail.

The introduction of antibiotic fermentations greatly stimulated interest in fermentation rates. It was recognized from the first that these processes were markedly different from most earlier fermentations. Studies of the chemical changes in penicillin biosynthesis required frequent analysis of carbohydrate and nitrogen levels, cell weight and antibiotic titre. From these, general rate patterns could be discerned and it was soon noted that the process comprised two more or less distinct phases; growth and antibiotic production.

Dulaney *et al.*,[8] noticed the same general behaviour in streptomycin fermentations. They defined an initial 'growth phase' in which mycelium was rapidly generated, accompanied by a reduction in soluble medium constituents (carbon, nitrogen, phosphorous), rapid sugar utilization and high oxygen demand. Virtually no streptomycin was produced. Following this was an 'autolytic phase', characterized by a marked drop in mycelial

weight, release of nitrogen and inorganic phosphorous to the medium, low oxygen demand and rapid antibiotic synthesis. All strains examined exhibited the same basic pattern and gross medium changes had little effect on it.

Calam, Driver and Bowers[6] were among the first to support these general observations with specific experiments. Penicillin fermentations were carried out at several temperatures between 12° and 32°C and average rates of growth, respiration and penicillin synthesis noted. By plotting the observed rates in the Arrhenius manner (logarithm of rate versus reciprocal absolute temperature) it was possible to characterize each process by the slope of the line obtained, the 'thermal increment'. Since these three rates all exhibited significantly different thermal increments, the authors concluded that the 'pace-setting enzyme-systems' involved are different.

Any survey of the literature on fermentation rates underscores the dearth of direct kinetic studies of this type. One cardinal reason for this is the matter of experimental procedure itself. Rate information can best be obtained in steady-state (continuous) systems with automatic control of process variables.

In an excellent example of this approach, Kempe, Gillies and West[15] studied rates of acid production by *Lactobacillus delbrueckii* at controlled pH. Rates were determined by differentiating the automatically recorded curve of alkali addition. Steady-state operation at various temperatures provided values for an Arrhenius-type plot which gave an activation energy of 17 kcal/g mole, a value in the range characteristic of many chemical reactions.

For one reason or another satisfactory methods for automatic regulation and control in fermentation studies have only recently been introduced and most experiments so far reported involve the classical batch technique. Data which permit the computation of rates are rare—and often inadequate because of the absence of key values. Of course the aim of these experiments was yield improvement in batch processes, not the gathering of kinetic data. Still, despite the inherent limitations of the unsteady-state, batch technique, a surprising amount of information has been accumulated and a great deal has been learned about the general kinetic aspects of various fermentation processes.

From an analysis of the rate patterns in batch alcohol, citric acid and penicillin fermentations, for example, Gaden[9] distinguished between three broad kinetic groups.

(1) Processes in which the desired products (ethanol, gluconic and lactic acids, for example) arise directly from oxidation of the primary carbohydrate.
(2) Processes in which the products (citric acid, for example), though also resulting from carbohydrate dissimilation, do so indirectly and accumulate only under conditions of restricted or abnormal metabolism.
(3) Processes in which product formation has no apparent association with carbohydrate oxidation (penicillin and many other antibiotics are examples of this type).

It must be recognized that a classification of this sort is based on purely empirical examination of batch fermentation results, not on a full and complete understanding of the individual mechanisms involved and their relationships to one another. Still, until such understanding has been achieved, empirical analysis is a powerful and useful tool—so long as its limitations are kept constantly in mind.

More recently Luedeking[16] investigated the kinetics of the lactic acid fermentation using a batch process at controlled pH. He showed that rate of product formation is indeed proportional to the rate of substrate utilization as expected. Furthermore, rates of acid production could be related to rates of growth by a simple expression involving two constants dependent on the pH of the fermentation.

Subsequently, the performance of single or multi-stage continuous lactic acid processes were predicted from these batch results by analytical and graphical methods.[17] Equations for both transient and steady-state operations of the continuous system have been developed.

Kinetic Phenomena in Fermentation

The first problems in studying fermentation kinetics are (1) the establishment of consistent rate expressions, and (2) the selection of meaningful rate processes to be measured.

Rates and Productivity

To avoid confusion, the term 'productivity' has been recommended for the time-average output of a process.[9] The expression 'fermentation rate' can then be reserved for the instantaneous rate of change of any concentration factor—sugar, product, cell weight, etc. These distinctions are shown graphically in Fig. 1.

Productivity is defined as the final product concentration divided by the time from inoculation to delivery of the batch. It might seem more reasonable to divide by the total process time from delivery of one batch to delivery of the next. This would include many operational factors involved in turnover of a tank,

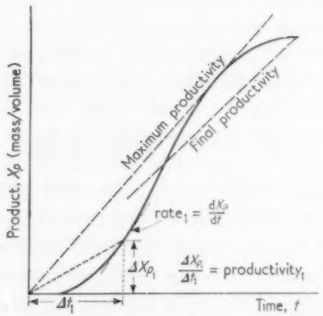

Fig. 1. Fermentation rates and productivity

like cleaning, batching and filling, which have little or nothing to do with the actual fermentation system. While it is essential for proper economic analysis of the plant, such an overall productivity has little use in analyzing the fermentation process itself.

Two bases for expressing fermentation rates have been proposed:[9]

(1) The *volumetric* rate, or the rate of change of concentration with time; its units are mass/unit time (unit volume).
(2) The *specific* rate, or the volumetric rate divided by cell concentration; its units are mass/unit time (unit cell mass).

The first is the preferred form for process design, especially for continuous systems, because it includes a volume term. The second is best for kinetic analysis because it puts everything on a comparable basis—unit mass of tissue. It does not follow, of course, that this unit tissue mass is physiologically identical throughout the fermentation process.

Rate process in fermentation

Rate measurements may be applied to an almost infinite number of factors in a fermentation system. Three of these, however, have been consistently singled out for study—growth, sugar utilization and product formation.

Growth is taken as a rough expression of the total catalytic activity in the system. Admittedly, tissue accumulation is only the crudest expression of the true levels of activity of the various enzyme systems involved. Until these can actually be determined, however, it is the best measure we have.

Synthetic processes require the metabolic energy released by oxidation of primary carbon sources and sugar utilization is generally taken as an indication of the rate of energy release to the system. While it is true that proteins and fats are similarly degraded, with accompanying energy release, carbohydrate sources are ordinarily the major energy suppliers. At the same time these materials are frequently the substrates from which specific products are formed. The key rate, product formation, needs no further elaboration.

Perhaps the greatest difficulty encountered in the examination of any complex fermentation process is the lack of any stoichiometric relationship between reactants and products. Lacking this, measurements of the three basic rates defined above may still be made. They offer the singular advantage of being determined directly from the measurements most commonly made in fermentation process studies, tissue mass, sugar and product concentrations.

Complete rate patterns, on both volumetric and specific bases, for a typical complex fermentation (streptomycin biosynthesis) are shown in Fig. 2. They were calculated from data of Sikyta *et al.*, in the manner previously described.[9]

Fermentation process variables

Primary process variables in fermentation are: (1) temperature, (2) pH, and (3) nutrient (or reactant) concentration (including oxygen). In addition certain conditions of the physical environment, like fluid turbulence and equipment design features which affect mass transfer in the reaction zone, must be considered.

Note that the fundamental composition of the nutrient environment, as opposed to the concentration of specific components (sugar, nitrogen sources, etc.), is not included. This is considered an inherent feature of the process system and not a 'process variable' in the usual sense. While this view is reasonable for most other chemical reaction systems, it may not be so for fermentation. One cannot synthesize ammonia unless the reaction mixture contains both nitrogen and hydrogen (the mole ratio of these reactants is the 'process variable') but tetracycline can be produced in a wide variety of nutrient media.

Fermentation Process Types

Fermentation processes may be classified in a number of different ways. The first systematic approach was proposed by Gale[11] who grouped microbiological processes in a series of type groups, oxidation, reduction, hydrolysis, etc. Such an arrangement though fundamentally attractive, is only suitable for specific reactions operating on specific substrates to yield specific products. Unfortunately, many commercially important fermentation processes cannot be so neatly described.

Gale's classification scheme has recently been extended by Stodola[24] and others,[25] who have proposed a more detailed breakdown of 'type reactions'. In this scheme, micro-organisms, or more specifically their enzyme complements, are looked at as added means for controlled organic synthesis. Again, this concept is not applicable to most of the fermentation processes now practised commercially—at least at the present level of knowledge regarding mechanisms.

A different approach was proposed by Gaden.[10] It is summarized in modified form in Table I. Here fermentation processes rather than specific reactions are grouped together and the overall free energy change involved is the basis for classification.

The primary advantage of this scheme is technological; it coincides with the general classification of fermentation rate patterns suggested earlier.[9] Experience has shown that fermentation processes fall more or less into three kinetic groups, which may be designated 'types I to III' for convenience. Their relationship to the general reaction types is shown in Table I and summarized below:

Type I: processes in which the main product appears as a result of primary energy metabolism. Examples of this type of system are most common in the older branches of fermentation technology, for instance: (1) aerobic yeast propagation (mass propagation of cells in general), (2) alcoholic fermentation, (3) oxidation of glucose to gluconic acid, and (4) dissimilation of sugar to lactic acid.

Type II: processes in which the main product arises indirectly from reactions of energy metabolism. In systems of this type the product is not a direct residue of oxidation of the carbon source but the result of some side-reaction or subsequent interaction between these direct metabolic products. Examples are: (1) formation of citric and itaconic acids, and (2) formation of certain amino acids.

Type III: processes in which the main product does not arise from energy metabolism at all but is independently elaborated or accumulated by the cells. It is perfectly true that carbon, nitrogen, etc., provided in essential metabolites appear in product molecules but the major products of energy metabolism are CO_2 and water. Antibiotic synthesis (Fig. 2) is a prime example of this type.

Table I. Fermentation Process Types

Dissimilation Reactions $\Delta F = -$	Biosynthesis $\Delta F = +$
Type I. Simple: $A \rightarrow$ products $A \rightarrow B \rightarrow C \rightarrow$ products	Type III. Biosynthesis of complex molecules: Polymerizations—carbohydrates, proteins Antibiotics, vitamins, etc. Fats
Type II. Complex: $A \rightarrow B \rightarrow C \rightarrow$ $\downarrow \quad \downarrow$ $D \quad E$ $\searrow \quad \swarrow$ $\downarrow$ products	

Each of these types demonstrates a fairly distinctive rate pattern. These are shown schematically in Fig. 3. The Type I processes show only one maximum for each of the rate processes and these are virtually coincident, hence the term 'growth-associated' often used for products of this process type.

In the Type II process two rate maxima are distinguishable. In the first phase tissue is produced with little product formation;

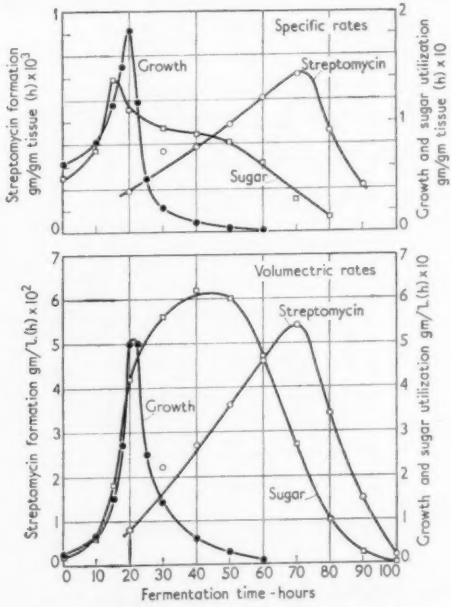

Fig. 2. Streptomycin fermentation rates

in the second product formation rate is maximized. Rapid carbohydrate utilization is common to both. Unfortunately, very few kinetic data are available for this group. In fact, until the recent development of microbiological processes for amino acids (probably Type II), the citric acid fermentation was the only example for which rate information had been published.

Type III processes again show two distinct phases. In the first tissue accumulation and all aspects of energy metabolism are

maximized with virtually no accumulation of the desired product; in the second oxidative metabolism is practically over and product accumulation is maximum. Both penicillin and streptomycin (Fig. 2) fermentations are excellent examples of the Type III kinetic pattern.

It must be emphasized that these are only generalizations for technological convenience. They are neither perfect nor comprehensive and great variations may occur. A particular fermenta-

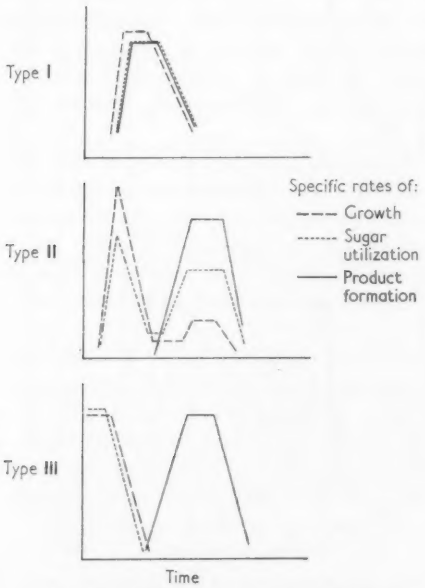

Fig. 3. Fermentation rate patterns

tion type may exhibit widely different behaviour with major changes in medium composition and process conditions. Strain variations, on the other hand, seem to have little effect on the general rate patterns.

Exceptions are found in all groups, especially Type III. In fact it may prove necessary to subdivide this group further as more kinetic information on complex processes becomes available.

One apparent exception is the production of oxytetracycline. Doskočil *et al.*[7] have presented a very complete study of metabolic changes observed during this fermentation. Rate curves calculated from these are shown in Fig. 4.

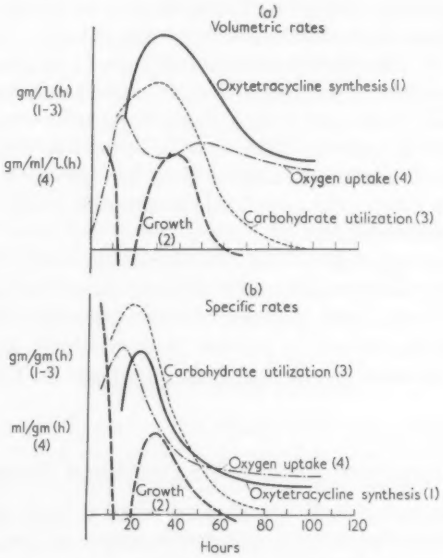

Fig. 4. Oxytetracycline fermentation rates

These authors[7] did not attempt any detailed analysis of rates, but they did suggest a multiphase nature for this process. Specifically they proposed five periods as follows:

(1) Lag: virtually no metabolic activity.

(2) Growth of primary mycelium: very high level of metabolism (respiration, nucleic acid synthesis, etc.), no antibiotic formation.

(3) Fragmentation of primary mycelium: respiration and nucleic acid synthesis fall, antibiotic synthesis is just starting.

(4) Growth of secondary mycelium; rapid antibiotic production, renewal of nucleic acid synthesis, further decrease in respiration.
(5) Stationary phase: no further growth, metabolic activity low but antibiotic synthesis continues.

Another process which one would expect to fall in Type III is the chloramphenicol (chloromycetin) fermentation. On the basis of very scanty data, however, it too appears to be an exception to the general pattern. If it is in fact, then the two processes which give a typical behaviour both involve organisms which normally fragment during growth. This may well lead to a characteristic kinetic behaviour different from that for streptomycin; unfortunately, the information available is not sufficiently complete to permit any firm conclusion.

A generous amount of sub-classification would undoubtedly remove most discrepancies. At the same time, however, it would make void the primary purpose of this approach—the establishment of certain reasonably reliable generalizations about fermentation rate patterns which can serve as a basis for further kinetic studies.

Fermentation Kinetics and Continuous Processes

Many reasons, both practically useful and intellectually satisfying, can be offered to justify more intensive study of fermentation kinetics but one outweighs all others: we cannot hope to operate continuous processes at a predictable steady state unless the relationships between major rate processes and the effects of process variables on them are known.

The reactor system which is apparently best adapted to continuous fermentation is the homogeneous, overflow type, with virtually complete backmixing. To establish an overflow reactor at steady state all rate processes must be in balance. It is possible to achieve this by simply letting the system hunt for such a point, but no one can predict in advance where this point will be. Such a procedure is hardly an adequate basis for plant operation.

For the 'kinetically simple' Type I fermentations, prediction of continuous steady state operating conditions from batch data is

theoretically possible.[13, 17, 18] Furthermore, this type of process can be operated satisfactorily in a single stage system, although additional stages may be added to ensure economical utilization of nutrients supplied. Both these points have been demonstrated experimentally in a number of cases.[5, 13, 17]

On the other hand, kinetic considerations alone demand at least two stages for satisfactory operation of the more complex process types (II and III). In the first, conditions will be adjusted to provide maximum rates of growth, and energy metabolism, in the second, for maximum product formation. Kinetic studies for continuous process design should therefore be aimed primarily at elucidating the relationships between these various rates and the major, controllable process variables.

The only complex fermentation process for which studies of this sort have been made is the biosynthesis of penicillin. In the final section of this paper, that information will be collected and related to illustrate the kinetic nature of the Type III process.

Penicillin Process Kinetics

Early attempts to clarify the effects of process variables on the two phases of the penicillin fermentation were seriously handicapped by the inadequacies of available experimental techniques. Even so a general picture was obtained. With improved procedures this has been greatly amplified over the last decade until the effects of major process variables on growth and antibiotic formation are reasonably understood. Temperature and pH are the best examples.

Temperature

Stefaniak *et al.*[23] found no effect on overall penicillin yields between 20° and 29°C with an early culture (X–1612). At 32°C, however, antibiotic yields fell while oxidative metabolic processes (sugar utilization, etc.) were more rapid.

In the work previously cited, Calam, Driver and Bowers[6] set the optimum temperatures for growth and penicillin formation at 30° and 25°C, respectively. These conclusions were arrived at rather indirectly because they did not, in fact, separate the two phases of the process experimentally.

This was done by Owens and Johnson[21] who showed that growth rates were highest around 30°C while penicillin synthesis proceeded most rapidly near 20°C. A two-stage fermentation with the temperature reduced from 30° to 20°C after 40 h gave the highest penicillin titre.

pH

The importance of pH in the penicillin fermentation was early recognized. Lacking reliable means for external control, most

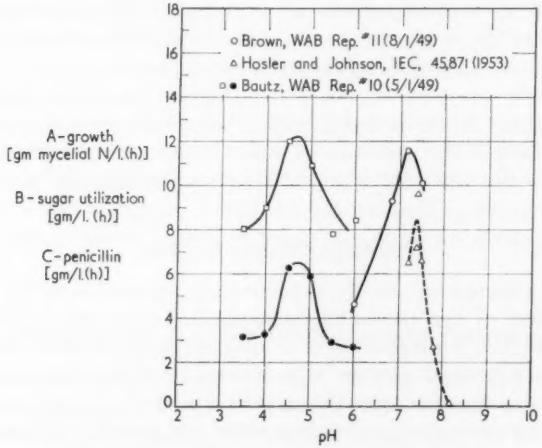

Fig. 5. pH effects in pencillin biosynthesis

processes employed medium formulations which provided a degree of internal buffering. A number of laboratory studies with externally controlled pH have been reported,[2, 3, 14] however, and the results are plotted on a common basis in Fig. 5. Note that the rates indicated are average rather than instantaneous. This does not alter the fundamental relationships shown.

From these experiments it is clear that the growth phase of the penicillin fermentation should be operated at a pH value around 4·5–5 while antibiotic formation will be maximized around 7–7·5.

It is also interesting to note the effect of external pH control on

rate patterns in a penicillin fermentation. Brown and Peterson[4] have reported batch fermentations employing a medium which tended to become alkaline. After 30 h, the pH was adjusted to 7·0 with acid and held there (approximately) by controlled acid addition. Volumetric and specific rate patterns calculated from their results are shown in Fig. 6. Since no determinations of

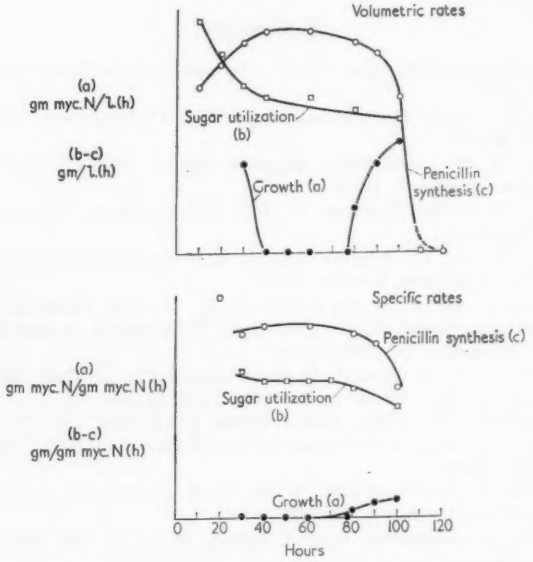

Fig. 6. Pencillin fermentation rates with pH control

mycelial nitrogen were made before the 30-h point, specific rates (based on mycelial nitrogen, not dry tissue in this case) cannot be computed for the early hours.

With pH control, constant rates of metabolism and product formation may be sustained for a long time, even in the unsteady-state batch process. Extended batch processes of this type may very well be practical competitors of continuous operations, particularly if the operating problems which have often been encountered in continuous systems prove difficult to overcome. The limit on such a process, assuming continuous nutrient addition as

well as pH control, will presumably be imposed by the accumulation of products toxic to the organism or inhibitory to its enzyme systems.

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References

1 Adams, S. L. and Hungate, R. E. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 1815

2 Bautz, M. *Antibiotics Research*, Report No. 10, University of Wisconsin (May 1, 1949)

3 Brown, W. E. *Antibiotics Research*, Report No. 11, University of Wisconsin (August 1, 1949)

4 Brown, W. E. and Peterson, W. H. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 1769

5 Butlin, K. R. *Continuous Culture of Microorganisms*, Czechoslovak Academy of Science, Prague (1958)

6 Calam, C. T., Driver, N. and Bowers, R. H. *J. Appl. Chem.*, 1 (1959), 209

7 Doskočil, J., Sikyta, B., Kašparová, J., Doskočilová, D. and Zajíček, J. *J. gen. Microbiol.*, 18 (1958), 302

8 Dulaney, E. L., Hodges, A. B. and Perlman, D. *J. Bact.*, 54 (1947), 1

9 Gaden, E. L., Jr. *Chem. & Ind.* (*Rev.*) (1955), 154

10 Gaden, E. L., Jr. *Chem. Engng.* (April, 1956), 159

11 Gale, E. F. *Chemical Activities of the Bacteria.* (1947). New York: Academic Press

12 Gastrock, E. A., Porges, N., Wells, P. A. and Moyer, A. J. *Industr. Engng. Chem.* (*Industr.*), 30 (1938), 782

13 Herbert, D., Elsworth, R. and Telling, R. C. *J. gen. Microbiol.*, 14 (1956), 601

14 Hosler, P. and Johnson, M. J. *Industr. Engng. Chem.* (*Industr.*), 45 (1953), 871

15 Kempe, L. L., Gillies, R. A. and West, R. E. *Appl. Microbiol.*, 4 (1956), 175

16 Luedeking, R., Ph.D. Thesis, Dept. of Chemical Engineering, University of Minnesota (1956)

17 Luedeking, R. and Pivet, E. L. This Journal, 1 (1959), 393

18 Maxon, W. D. *Appl. Microbiol.*, 3 (1955), 110

19 Moyer, A. J., Wells, P. A., Stubbs, J. J., Herrick, H. T. and May, O. E. *Industr. Engng. Chem.* (*Industr.*), 29 (1937), 777

20 Moyer, A. J., Umberger, A. J. and Stubbs, J. J. *Industr. Engng. Chem.* (*Industr.*), 32 (1940), 1379

21 Owens, S. P. and Johnson, M. J. *Appl. Microbiol.*, 3 (1955), 375

22 Sikyta, B., Doskočil, J. and Kašparová, J. This Journal, 1 (1959), 379

[23] Stefaniak, J. J., Gailey, F. B., Jarvis, F. G. and Johnson, M. J. *J. Bact.*, 52 (1946), 119
[24] Stodola, F. H. *Chemical Transformations by Microorganisms.* (1958). New York; Wiley
[25] Wallen, L. L., Stodola, F. H. and Jackson, R. W. *Type Reactions in Fermentation Chemistry.* (1959). U.S. Dept. of Agriculture
[26] Wells, P. A., Moyer, A. J., Stubbs, J. J., Herrick, H. T. and May, O. E. *Industr. Engng. Chem.* (*Industr.*), 29 (1937), 653

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Transient and Steady States in Continuous Fermentation. Theory and Experiment*

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Summary. Steady-state and transient equations are derived for continuous-flow fermentation processes. Analytical as well as graphical methods for predicting from batch data the performance of single- or multi-stage continuous fermentations are developed. The criteria for stability are also examined. Actual experimental measurements of continuous lactic acid fermentations conducted at controlled pH levels agree with the theory developed.

Introduction

The efficiencies and advantages characteristic of a continuous process in contrast to batch operation,[10,14,27] make continuous fermentation a logical goal for fermentation industries.[9,19] Continuous fermentations may be conducted to produce micro-organisms efficiently and rapidly,[6] to obtain one or more of the products of the fermentation,[5] or as a laboratory tool in the study of the physiology, metabolism, or genetics of micro-organisms.[21,23]

Contamination and culture degeneration need no longer be considered insurmountable difficulties in employing continuous fermentation industrially.[9] Elsworth and Meakin,[4] for example, have conducted a pilot plant fermentation with *Aerobacter aerogenes* continuously for 2,600 h. However, the lack of basic kinetic information and the lack of suitable design techniques are important.

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Methods have been developed in recent years in chemical engineering for designing or predicting the performance of single- or multi-stage continuous stirred tank reactor systems from the batch reaction kinetic data.[2,3,14,27] This theory is extended herein to the special case of fermentations, and the equations and graphical procedures are then confirmed with batch and continuous operation data on the lactic acid fermentation.

Monod[21] has derived the steady-state equations for a continuous fermentation where the growth rate is controlled by nutrient concentration, and has experimentally demonstrated in extremely dilute broths the stability of that steady state. Finn and Wilson[6] have considered a continuous fermentation where the organisms grow logarithmically, and they have accounted for the interesting steady cycling of population sometimes encountered in terms of an incompletely buffered medium and a metabolic lag in response to pH changes.

Theoretical equations for continuous fermentation with two fermentors in series have been derived by Golle[10] to describe the steady-state populations, and also for the filling of the empty second vessel from the first vessel. Golle[10] also considered the cases where contaminants and mutants might replace the desired organisms. Novick and Szilard[23] derived equations describing the rate of development of genetic mutants in continuous fermentation. A simple method of calculating cycle times for continuous fermentations from batch data was described by Adams and Hungate.[1] Maxon[19] has reviewed continuous fermentation applications and theory.

Continuous Fermentation Theory

Consider first a single stirred-tank reactor (CSTR) in which a continuous fermentation is being conducted, and where the agitation is sufficient to assure homogeneous conditions so that the composition of the effluent from the vessel is always the same as that in the vessel. This is the assumption usually applied to CSTR systems[6,10,14,18,19] and is readily attainable in them.[17]

The material balance over the vessel with respect to the bacterial mass is

$$\begin{bmatrix}\text{Change within}\\ \text{the vessel}\end{bmatrix} = \begin{bmatrix}\text{Increase}\\ \text{due to}\\ \text{the feed}\end{bmatrix} + \begin{bmatrix}\text{Increase}\\ \text{due to}\\ \text{growth}\end{bmatrix} - \begin{bmatrix}\text{Decrease due}\\ \text{to loss in}\\ \text{effluent}\end{bmatrix}$$

$$\frac{dN}{dt} = N_0\frac{F}{V} + \left(\frac{dN}{dt}\right)_G - N\frac{F}{V} \tag{1}$$

where

N = mass concentration of organisms in the vessel, the bacterial density, mg/ml
N_0 = mass concentration of organisms in the feed, mg/ml
t = time, h
$\left(\frac{dN}{dt}\right)_G$ = growth rate of the organisms, mg/ml h
F = volumetric flow rate, ml/h
V = volume of liquid in the vessel, ml

$(dN/dt)_G$ designates the rate of change of bacterial density due only to growth, while (dN/dt) represents the net rate of change of bacterial density in the reactor from all causes, as under transient conditions.

The specific growth rate k, represents the rate of increase of bacterial protoplasm per unit of bacterial protoplasm present, i.e.,

$$k = \frac{1}{N}\left(\frac{dN}{dt}\right)_G \tag{2}$$

which is at times referred to as the Naperian growth rate[21] or logarithmic growth coefficient. In batch fermentations k may conveniently be determined by measuring the slope of the curve obtained when the natural logarithm of the bacterial density is plotted against time.

The average or nominal holding time θ, is defined as V/F. Then, when the feed is sterile ($N_0 = 0$), equation (1) becomes:

$$\frac{dN}{dt} = \left(k - \frac{1}{\theta}\right)N \tag{3}$$

For steady-state operation in a single vessel, $(dN/dt) = 0$, and by equation (3), the holding time equals the reciprocal of the specific growth rate of the bacteria,[6, 10, 19, 21]

$$\theta = \frac{1}{k} \tag{4}$$

Logarithmic Growth

Transient and steady-state equations are now developed for continuous operation within the region where, in the batch process, the specific growth rate is constant, i.e., $k = k_c$.

Assume a fermentation where a substrate is converted almost entirely to a single product, such as in the dissimilation of glucose to lactic acid by a homofermentative organism. The development will be equally valid for cases where the substrate is converted to several products, provided the products are always formed in a constant ratio to one another.[2] At constant pH the rate of lactic acid formation for the entire batch fermentation (except possibly for the lag phase and at the very end of the fermentation) has been related[16] to growth rate and to bacterial density by the expression

$$\left(\frac{dP}{dt}\right)_G = \alpha\left(\frac{dN}{dt}\right)_G + \beta N \tag{5}$$

where

P = product concentration, mg/ml

$\left(\frac{dP}{dt}\right)$ = rate of product formation due to fermentation, mg/ml h

α, β = fermentation constants fixed by the organism, substrate, pH, and temperature.

From equation (5), since $k = k_c$, a constant,

$$\left(\frac{dP}{dt}\right)_G = \gamma\left(\frac{dN}{dt}\right)_G \tag{6}$$

where γ is a constant of proportionality. During this phase the rate of acid production remains proportional to the rate of growth.

From equations (5), (2) and (6),

$$\gamma = \alpha + \frac{\beta}{k_c} \tag{7}$$

Further, for the lactic acid fermentation and some other fermentations it can be assumed that the rate of product formation is proportional to the rate of substrate utilization:

$$\left(\frac{dP}{dt}\right)_G = -p\left(\frac{dS}{dt}\right)_G \tag{8}$$

where

S = the concentration of the substrate, usually a sugar such as glucose, mg/ml

p = efficiency of the fermentation process expressed as the ratio of product formed to substrate consumed. This ratio may be derived from the per cent conversion[15]

$-\left(\frac{dS}{dt}\right)_G$ = the rate at which substrate is consumed by the organisms, mg/ml h.

For continuous fermentation in a single vessel in the logarithmic phase only, three simultaneous differential equations are obtained by combining mass balances with equations (6) and (8):

$$\frac{dN}{dt} = \left(k_c - \frac{1}{\theta}\right)N \qquad \text{(bacterial mass balance)} \quad (9)$$

$$\frac{dP}{dt} = \gamma k_c N - \frac{P}{\theta} \qquad \text{(product mass balance)} \quad (10)$$

$$\frac{dS}{dt} = \frac{S_0}{\theta} - \frac{\gamma k_c N}{p} - \frac{S}{\theta} \qquad \text{(substrate mass balance)} \quad (11)$$

where S_0 is the concentration of substrate in the feed, mg/ml.

At the steady state there is no change with time in bacterial density, nor in the product or substrate concentrations, i.e., $(dN/dt) = 0$, $(dP/dt) = 0$ and $(dS/dt) = 0$. Therefore, from transient equations (10) and (11) using equation (7) or (4), two equations describing the steady-state concentrations are obtained:

$$P = \gamma N \quad (12)$$

$$S = S_0 - \gamma N/P \quad (13)$$

Although they are derived for the steady state, equations (12) and (13) will later be shown to apply also under certain conditions to transient operation.

In the logarithmic growth region the steady-state value of bacterial density N is not determined solely by operating conditions, but also by the initial value of bacterial density. In fact, the equations indicate that N can be held at any value within the range of logarithmic growth when the flow is adjusted so that $1/\theta = k_c$. When continuous flow at a rate $F = Vk_c$ is begun,

the bacterial density remains constant at its initial value if the fermentation is within the logarithmic growth phase.[6]

Integration of equations (9), (10) and (11) yields a set of three general equations for transient operation in a continuous-flow stirred-tank reactor:

$$N = N_i e^{(k_e - 1/\theta)t} \tag{14}$$

$$P = P_i e^{-t/\theta} + \gamma N_i e^{-t/\theta}(e^{k_e t} - 1) \tag{15}$$

$$S = S_0(1 - e^{-t/\theta}) + S_i e^{-t/\theta} + \frac{\gamma N_i}{p}(e^{-t/\theta})(1 - e^{k_e t}) \tag{16}$$

These equations describe the concentrations of bacteria, product and substrate, in a continuous fermentation at time t, where the initial concentrations at $t = 0$ are indicated by the subscript i. The two conversion ratios, γ and p, are assumed constant. All this, it must be remembered, is for processes within the range of logarithmic growth.

The general transient equations (15) and (16) simplify to the steady-state relations (12) and (13) when the changes in concentration which have occurred and are occurring in the fermentation are due only to the growth of the bacteria during either batch or continuous operation, and to the effects of flow during continuous operation. For example, batch and continuous flow operation may be alternated and the simplification remain valid.

To demonstrate this the batch fermentation will first be considered. Equations (6) and (8) apply to fermentation processes in either batch or continuous operation so long as the specific growth rate is constant. Integration of these two equations gives $P = \gamma N + C$, and $S = -ps + C'$, where C and C' are constants of integration. Where initial concentrations of bacteria and product are negligibly small and the early deviations from logarithmic growth may be neglected, and where the initial substrate concentration before inoculation is S_0, the above integrated equations become identical to equations (12) and (13), $P = \gamma N$, and $S = S_0 - \gamma N/p$.

When continuous operation is begun at some point during the batch fermentation, the initial concentrations for the transient operation are given by equations (12) and (13). Hence, the initial concentrations to be used in the general transient equations (15) and (16) are $P_i = \gamma N_i$, and $S_i = S_0 - \gamma N_i/p$. When this is

done it follows from an algebraic rearrangement and from equation (14) that the two general transient equations of concentration simplify to $P = \gamma N$, and $S = S_0 - \gamma N/p$, identical to the steady-state equations (12) and (13).

However, in continuous fermentations where any of the concentrations have been caused to change at any time by processes other than those of normal bacterial growth and continuous flow operation, the general transient equations for concentrations, equations (15) and (16), must be used. They should be used, for example, if at any time some organisms from the effluent had been concentrated by centrifugation and then recycled into the fermentation, or if an extra quantity of nutrient or product had been added during the fermentation at any time.

One more special transient case will be considered. Assume that during steady-state operation in the logarithmic phase the product or the substrate concentration is suddenly altered, but that flow rate and bacterial density remain the same. The system continues at steady state with respect to bacterial density, but as a result of the alterations it becomes transient in product concentration or substrate concentration or both. Let P_t and S_t be the new initial values of P and S. Since $1/\theta = k_c$, equations are readily derived from equations (15) and (16) to express the concentrations P and S as simple functions of time t and the bacterial density N:

$$P = P_t e^{-t/\theta} + \gamma N(1 - e^{-t/\theta}) \tag{17}$$

$$S = S_0 - \gamma N/p - (S_0 - \gamma N/p - S_t)e^{-t/\theta} \tag{18}$$

It is assumed in deriving these equations that neither the change in substrate concentration nor the change in product concentration affects the specific growth rate k_c. In the logarithmic phase substrate concentration does not ordinarily affect growth rate; only where the substrate concentration is very low is growth rate affected.[21] Changes in product concentration may or may not affect the growth rate.

Growth Limited by Toxic Product Formation

For continuous fermentation the region of greatest interest will most often lie beyond the logarithmic growth phase where the

specific growth rate k is no longer constant. From an early maximum (which is usually identical with the logarithmic growth constant k_c), specific growth rate declines until near the end of the fermentation it approaches zero, and growth ceases.[16]

Two explanations for the decline are commonly offered. Growth may slow and finally cease altogether because the nutrient substance is exhausted, or because toxic products formed by the fermentation accumulate.[12] Monod [21] has developed equations to describe continuous operation where specific growth rate is controlled by the concentration of the substrate. In the following, continuous-fermentation equations are derived for the alternate case where the toxic products of the fermentation limit the growth.

Assume that the specific growth rate is diminished by the action of the toxic products as a linear function of their concentration.[12]

$$k = k_m - qP_t \tag{19}$$

where P_t is the mass concentration of toxic products of the fermentation, mg/ml, and k_m is the maximum specific growth rate attained in the fermentation, h^{-1}.

Moreover, assume that the rate of formation of the toxic products may be described by an equation analogous to the experimental equation (5).

$$\left(\frac{dP_t}{dt}\right)_G = \alpha_t\left(\frac{dN}{dt}\right)_G + \beta_t N \tag{20}$$

where the subscript t designates toxic products.

For continuous fermentation in a single vessel, two simultaneous rate equations are derived by combining equations (19) and (20) with mass balances for the bacteria and the toxic product.

$$\frac{dN}{dt} = (k_m - qP_t)N - N/\theta \tag{21}$$

$$\frac{dP_t}{dt} = \left[\alpha_t(k_m - qP_t) + \beta_t\right]N - P_t/\theta \tag{22}$$

For the steady state in such a continuous fermentation, the above transient equations reduce to:

$$P_{ts} = (k_m - 1/\theta)/q \tag{23}$$

and

$$N_s = P_{ts}/(\alpha_t + \beta_t\theta) \tag{24}$$

where the subscript s refers to the steady state.

That the steady state described by these equations is a stable equilibrium may be demonstrated by assuming that N has been increased slightly above the steady-state value given by equation (24). It can be seen from equation (22) that the increase in N results in a higher rate of formation of the toxic product, and P_t is thereby increased above its steady-state value, too. This increase of P_t, on the other hand, makes dN/dt negative (see equation (21)) so that N begins to decrease and return towards its original steady-state value. By equation (22) this decrease in N causes dP_t/dt to begin to return to zero, and therefore P_t also tends to be restored to its steady-state value. Thus the system possesses the characteristics of stable equilibria in that it tends to return to the original steady state after a displacement from it. The stability of this system has also been proved rigorously.[15]

Since Monod[21] has demonstrated a stable equilibrium for continuous fermentation where nutrient concentration controls the growth rate, it is apparent that no matter which of the two major assumptions concerning growth is used, a stable equilibrium will result in the idealized continuous fermentation beyond the region of logarithmic growth.

Continuous Fermentation in a Series of Vessels

In this case the overflow of effluent from one vessel constitutes the feed to the following vessel. At steady state, a bacterial mass balance over the second vessel shows that

$$N_2 = N_1F/(F - k_2V_2) \tag{25}$$

where

N_1 = bacterial density in the first vessel, mg/ml
N_2 = bacterial density in the second vessel, mg/ml
F = volumetric flow rate, ml/h
k_2 = the specific growth rate of the organisms in the second vessel, h^{-1}
V_2 = liquid volume in the second vessel, ml.

The subscripts 1, 2, 3, ..., n, are used to denote conditions in the 1st, 2nd, 3rd, ..., nth, vessels respectively.

For a third fermentor,

$$N_3 = N_2F/(F - k_3V_3) \tag{26}$$

and for the nth fermentor,

$$N_n = N_{n-1}F/(F - k_n V_n) \tag{27}$$

By the successive application of equations (25), (26) and (27), the steady-state bacterial density in the last or nth vessel of the series in terms of the bacterial density in the first vessel is found to be

$$N_n = \frac{N_1 F^{n-1}}{(F - k_2 V_2)(F - k_3 V_3)\dots(F - k_n V_n)} \tag{28}$$

If the volumes are identical,

$$\frac{F}{V_1} = \frac{F}{V_2} = \frac{F}{V_3} = \dots = \frac{F}{V_n} = \frac{1}{\theta}$$

where θ is the nominal holding time. For this case equation (28) simplifies to

$$N_n = \frac{N_1(1/\theta)^{n-1}}{\left(\frac{1}{\theta} - k_2\right)\left(\frac{1}{\theta} - k_3\right)\dots\left(\frac{1}{\theta} - k_n\right)} \tag{29}$$

and since by equation (4), $1/\theta = k_1$,

$$N_n = \frac{N_1(1/\theta)^{n-1}}{(k_1 - k_2)(k_1 - k_3)\dots(k_1 - k_n)} \tag{29a}$$

For continuous fermentation in a series of equi-volume vessels, equation (29) or (29a) expresses the bacterial density in any vessel as a function of the bacterial density in the first vessel, the nominal holding time, and the specific growth rate of the organisms in each vessel.

From the standpoint of stability in equations (28) and (29), solutions for the bacterial densities in any of the vessels can be obtained only if $k_1 > k_2$, $k_1 > k_3$, $k_1 > k_4$, ..., $k_1 > k_n$.

If the specific growth rate k, in any vessel equals the specific growth rate in the next vessel of the series, both vessels are operating within a phase of logarithmic growth. In equation (29a), N_n becomes infinitely large for equal growth rates and no solution is possible. Furthermore, it will become evident from the discussion of graphical methods in the next section that to specifiy equal volumes in a multi-vessel continuous fermentation is

incompatible with a specification of equal specific growth rates in the vessels.

This does not deny, however, the possibility of conducting a continuous fermentation wholly within the region of logarithmic growth in a series of vessels. By equations (25), (26) and (27), such operation might be possible if $1/\theta$ in all of the subsequent vessels were greater than $1/\theta_1$. In other words the volume of the first vessel must be greater than any of the subsequent vessels. Even meeting this requirement does not assure success, however, since without external controls a stable equilibrium may not prevail in the first vessel. The question of stability will also be examined below using graphical methods.

Graphical Methods

Since the specific growth rate k, is usually a complex function of fermentation time and conditions, graphical methods, rather than analytical approaches, are most advantageously used to predict the performance and stability of continuous fermentation. For chemical reactions in continuous stirred-tank systems appropriate graphical methods have been described[2, 3, 14, 27] and are extended here to the fermentation problem. The assumptions expressed by equations (6) and (8) are unnecessary for the graphical procedure.

Graphical Solution for a Single Vessel

The first step of the graphical method is to plot the batch data of the growth rate $(dN/dt)_G$ against the bacterial density N for the entire cycle of positive growth. An experimental curve[16] for a lactic acid fermentation conducted batchwise at pH 6·0 is used to illustrate the method in Fig. 1.

The portion of the curve between points a and b represents the lag phase, the section between b and c the logarithmic phase, and the portion between c and e the phase of deceleration. The properties of the plot are such that the portion of the curve representing the logarithmic phase is a straight line which goes through the origin and whose slope is the logarithmic growth constant, k_c. Also, the maximum rate of growth, shown at

point d in Fig. 1, is attained during the phase of deceleration, some time after the end of the logarithmic growth phase where the specific growth rate is a maximum.

In a continuous fermentation the rate of loss of organisms is directly proportional to the concentration of organisms,

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = \frac{F}{V}N = \frac{N}{\theta}$$

Thus, $1/\theta$ is a constant of proportionality. On the plot, therefore, this loss is represented by a straight line of slope $1/\theta$, which appears

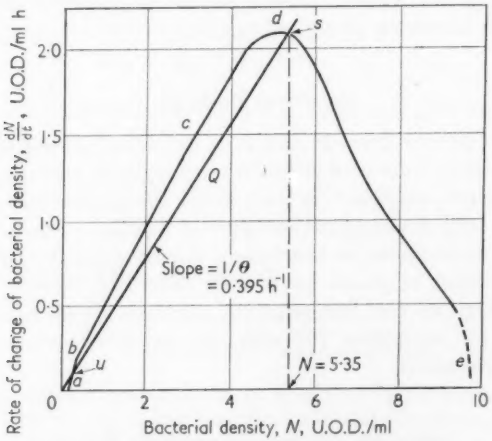

Fig. 1. Graphical solution for continuous lactic acid fermentation at pH 6·0 using a holding time (θ) of 2·53 h. A steady-state bacterial density of 5·35 U.O.D. per ml is predicted

on Fig. 1 as Q, the operating line. When there are no organisms in the feed, the operating line passes through the origin.

Wherever the operating line intersects the growth curve, loss is exactly compensated by growth, and an equilibrium is thereby described. By the graphical method, the steady-state population may be determined for any given holding time, or the necessary holding time may be determined for any desired population level.

In Fig. 1, the steady-state bacterial density is predicted for a continuous fermentation which is conducted using a nominal holding time of 2·53 h. The operating line is drawn with a slope

of 1/2·53 or 0·395 h^{-1}. Of the two intersections of the operating line with the growth curve, the lower at point u corresponds to an unstable equilibrium for reasons to be discussed later. Attention is given to the intersection at point s, which occurs at a bacterial density of 5·35 U.O.D./ml and accordingly is the steady-state value predicted. In a continuous fermentation conducted under these same experimental conditions of pH, temperature, medium, and organism, the actual value of the bacterial density at steady state was 5·47 U.O.D./ml, close to the predicted 5·35.

If only one vessel is being considered, one can, of course, predict holding times or bacterial densities equally well using curves of specific growth plotted against bacterial density. However, for continuous fermentations involving two or more vessels in a series, the graphical method described and illustrated above proves far easier to use.

The rate of change of the bacterial density in the vessel at any given level is represented on the graph by the vertical distance *from* the operating line to the growth curve. When the growth curve lies below the operating line, the rate of change (dN/dt), is negative, and the bacteria are being lost in the effluent faster than they reproduce.

When the operating line coincides with the abscissa, a batch fermentation is described, for the slope is zero, the holding time is therefore infinite and there is no flow. When the continuous fermentation is operated with such a short holding time that the slope $1/\theta$ of the operating line is greater than the logarithmic growth constant k_e, the operating line lies entirely above the growth curve and does not intersect it at any point. Under these circumstances the organisms are always washed out of the fermentor faster than they can proliferate. Thus continuous fermentation requires that $k_e \geqq 1/\theta > 0$.

Graphical Solution for a Series of Continuous Fermentors

The graphical method is easily extended to the case where a series of CSTR fermentors are considered (Fig. 2). Conditions for the first vessel are determined as in a single vessel. Now, however, the effluent from the first vessel is the feed for the second vessel and is of the same bacterial density as the contents of the

first vessel, N_1. The base of the operating line for the second vessel then is the point, $N = N_1$, $dN/dt = 0$, and the slope of the line is F/V_2, or $1/\theta_2$, where the subscript 2 denotes the second vessel. N_2 is the bacterial density in the second vessel, and therefore of the feed to the third vessel. The process may be repeated for as many times as there are fermentors in the series. In the example of Fig. 2, the bacterial density in the final vessel is N_3.

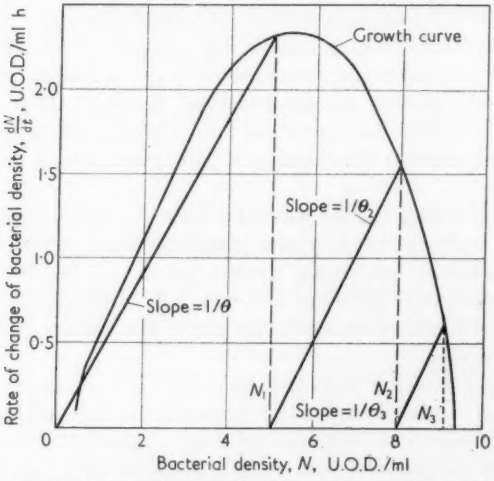

Fig. 2. Example of a graphical solution for continuous fermentation using three vessels in series

If the liquid volumes in all of the tanks are equal, all operating lines on the graph are parallel with a slope of $1/\theta = F/V_1 = F/V_2 = \ldots = F/V_n$. Where the volumes are unequal, the holding time varies from tank to tank since the flow rate is the same through the whole series, and the operating lines therefore are no longer parallel.

The important point to be observed concerning the graphical method is the ease with which one may use it to design the optimum operation of a continuous fermentation given only the batch data. A wide variety of possible arrangements can be quickly and easily considered in trial and error solutions, using many different combinations of holding times and vessel numbers.

One can easily work backwards from a desired bacterial density to determine operating conditions. If a recycle of organisms is contemplated, the starting point of the first operating line on the graph is moved along the abscissa from zero to the appropriate N_0.

In a continuous fermentation high flow rates are sought in order to process raw material as rapidly as possible, but at the same time high bacterial densities are desired for fermenting the materials as completely as possible. It is apparent from Fig. 1 that a single vessel does not combine the two desired conditions of high flow rate with high bacterial density. Hence the use of multi-vessel continuous fermentation systems.[9,10] When a second and a third vessel are added, a high bacterial density is obtained without a decrease of the flow rate, Fig. 2. By the graphical method one can usually ascertain at a glance whether the placing of another vessel in the series yields sufficient advantage to justify the additional cost. In the example of Fig. 2, a fourth vessel would result in a slightly higher bacterial density, but the increase from a fifth vessel would be slight indeed. Caution should be exercised in the region where the growth approaches the abscissa, since small errors are magnified and death is becoming a factor as the stationary phase is approached.

Stable and Unstable Equilibria

With this graphical method the question of stability is easily answered. In Fig. 1 it was observed that the operating line Q intersects the growth curve at u and at s, indicating that two equilibria are theoretically possible. The first intersection at u occurs during the lag phase, and this can be demonstrated to yield an unstable equilibrium in the following way. If the bacterial level is increased slightly from this equilibrium value, the growth curve lies *above* the operating line, i.e., the rate of growth is greater than the rate of loss of organisms in the effluent. Consequently, the bacterial density continues to increase rather than to return to the equilibrium point, and the equilibrium therefore must be considered to have been unstable.

On the other hand, at the second intersection s, the indicated steady state can by the same test be shown to be a stable equilibrium. If the bacterial density is increased slightly from this point, it will be seen from Fig. 1 that the operating line now lies

above the growth curve. Loss, therefore, exceeds growth, the population tends to decrease and the system is thereby returned to the equilibrium point at s.

Continuous operation within the logarithmic growth region constitutes a unique case in that if an equilibrium is sought here in accordance with equation (4), ($dN/dt = 0$) by setting the flow rate such that $1/\theta = k_c$, no single bacterial density is specified thereby.[6] Instead, any bacterial density within the logarithmic phase is possible and the equilibrium is only quasi-stable. If the bacterial density is suddenly changed to a new level, the system will continue constant at that new level, provided, of course, that it is still within the range of logarithmic growth and the flow rate is unchanged.

The graphical solution such as is shown in Fig. 1 assists in understanding the properties of this quasi-stable equilibrium. Since $1/\theta$ equals k_c, the operating line Q coincides with the entire section of the growth curve representing the phase of logarithmic growth, showing that all bacterial densities in this region are possible. When the feed rate is such that $1/\theta$ differs slightly from k_c, the plot shows that the system will drift slowly from its original value. It is this characteristic of the quasi-stable equilibrium that necessitates constant adjustment of the feed rate in order to maintain the bacterial density at steady state within the logarithmic phase. This, of course, could be accomplished by an automatic control varying the incoming flow rate.

In Fig. 1, if the operating line intersects the growth curve anywhere between points c and e, a stable equilibrium results according to the test described above. Since in most fermentations the specific growth rate declines steadily from the end of the logarithmic phase until the end of the fermentation (as in Fig. 1), the graphical solution indicates that a stable equilibrium results when most fermentations are conducted continuously in this range.

Experimental Methods for Continuous Fermentation

The experimental apparatus, Fig. 3, and procedures used in the kinetic studies of the lactic acid fermentation conducted batchwise[16] were modified somewhat for these continuous flow experiments. However, the same organism, *Lactobacillus delbrueckii*

NRRL–B445, was used with the same medium, an aqueous solution containing 5 per cent glucose and 3 per cent yeast extract with added mineral salts. All fermentations were conducted at 45°C, and the same means of automatic pH control with 2·5 N sodium carbonate solution were employed.

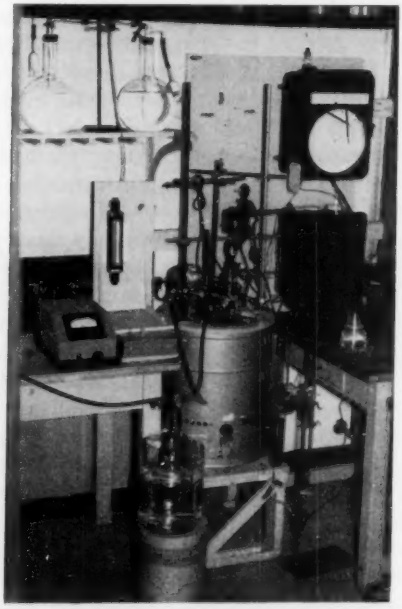

Fig. 3. General view of experimental assembly for continuous fermentations

Two 6-l. Florence flasks served as reservoirs for the sterilized medium, which was fed by gravity into the reactor through Tygon and glass tubing. Flow was regulated by a small stainless steel Hoke needle valve, and the rate of flow was measured by a Fischer and Porter Tri-flat rotameter, using the sapphire ball float. The rotameter was recalibrated for each run. All air inlets to the reservoir flasks contained filters of sterile cotton.

After each run the line from the reservoirs to the fermentor, including the needle valve and the rotameter, was rinsed well with

distilled water after which a quaternary ammonium solution was allowed to flow slowly through the system for a day. Finally, the line was rinsed again with about 10 l. of sterile water, and additional sterile distilled water was allowed to flow slowly through the system at rates less than 50 ml/h until the next run. The above measures essentially prevented contamination, although there was an indication from the plate counts that some slight contamination was present in three of the runs after several days of continuous operation. In all cases, however, the significant data had been obtained before there was evidence of contamination.

During continuous operation the fermented medium was continuously withdrawn from the fermentor through a capillary tube and then through rubber tubing to a 5-gal carboy receiver maintained under partial vacuum. A three-way cock permitted the taking of samples through the same vacuum overflow line. The entire arrangement for overflow and sampling is similar to one used in conjunction with a continuous Waldhof fermentor.[11]

The fermentor was well-baffled and a four-bladed agitator rotating at 300 rev/min provided the mixing. At this speed a slug of dye injected into the fermentor was observed to be completely dispersed in less than 20 sec. Since holding times were between 2 and 7 h, mixing may be considered to be quite adequate for homogeneity.[17] Samples obtained through the overflow and directly from the fermentor at the same time by a pipette, always measured the same in bacterial density. Foaming was prevented by adding small quantities of Dow-Cornering Antifoam AS–10, whenever needed. In preliminary tests the antifoam had no detectable effect on the fermentations at any concentration.

The medium was essentially sterilized in the fermentor and reservoir by bubbling in live steam while holding them at 100°C. The same methods of measuring bacterial density by optical density were used here as in the earlier studies.[16]

Samples for lactic acid or glucose analysis were collected in a sampling flask immersed in ice in order to minimize fermentative changes during the time of collection. Lactic acid was again analysed by the method of Friedemann and Graeser[7] incorporating modifications suggested by Smith.[16,25] In runs 1 to 4 the Hodge and Davis modification[13] of the Somogyi semi-micro method for

reducing sugars was followed. In the remaining runs when the glucose concentration was greater than about 10 mg/ml, the Munson-Walker method of analysis was used.[13] For glucose concentrations of less than about 10 mg/ml, glucose was determined by the colorimetric scheme described by Somogyi[26] using the chromogenic reagent of Nelson.[22]

Measurements, including glucose and lactic acid analyses were made in a series of continuous fermentations conducted at controlled pH levels. To obtain the total flow rate for these experiments, the rate of alkali addition was added to the flow rate of sterile medium. Correction was also made for the dilution effect of the added alkali on the glucose concentration of the feed stream. In runs 3, 4, 5 and 6 the periods of continuous flow operation were interrupted by periods of batch operation.

Discussion of Results

Test of a Basic Assumption

A most significant assumption made when continuous fermentation performance is predicted from batch data is that the organisms in the continuous fermentation are physiologically identical in their growth rates and metabolic activities with the organisms of the corresponding batch fermentation at the same population. This assumption might be invalid if, for example, the organisms are genetically unstable at high population levels, or if the generation time of the individual organisms in the general population might to some extent be an inherited characteristic. In the latter case, continuous operation would tend to select the faster growing cells. Powell's experiments[25] with *Bact. aerogenes*, *Strep. faecalis*, and *B. mycoides* show no correlation between the generation times of mother and daughter cells, thus indicating in these cases at least that selection will not occur in continuous culture. Experiments by Adams and Hungate[1] with a continuous alcoholic fermentation suggest that the assumption may not hold sometimes where the medium is nutritionally inadequate or where toxic materials are present. More work is needed in this area.

Nevertheless, the fact that predicted continuous performances are verified experimentally in this study confirms the validity of the assumptions for continuous operation over a period of a day.

One further test can be made by allowing a fermentation to go batchwise to completion after a period of continuous operation has ended. If the assumption holds, it should compare exactly to the corresponding batch fermentation.[1] In run C–1 with the pH controlled at 5·6, continuous operation was maintained for 6·4 h before the flow of fresh medium was stopped and the fermentation permitted to proceed batchwise again. Corrections were made for sampling and dilution.

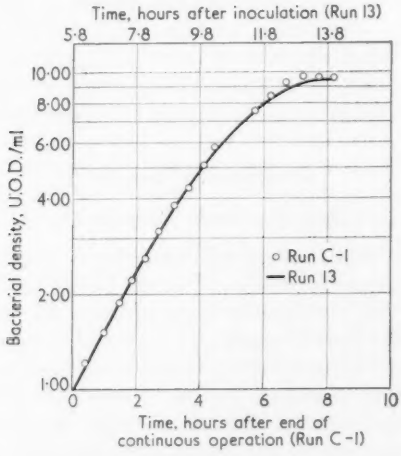

Fig. 4. Comparison of bacterial growth during two fermentations with pH controlled at 5·6. Run 13, represented by the solid line, was an ordinary batch fermentation.[16] The portion of run C–1 shown here represents batch fermentation begun after a period of continuous operation

The bacterial densities for this batch portion of run C–1 are plotted semi-logarithmically against time in Fig. 4. In order to compare the results to the batch experiment, part of the corresponding curve for a batch fermentation[16] at the same pH is superimposed on the graph with the time axis so placed that the bacterial densities are the same at the beginning of the comparison period. This corresponds to 5·18 h after the start of the batch fermentation. It is evident from Fig. 4 that there is little or no difference in the

progress of the two fermentations, thus further substantiating the original assumption that the organisms are physiologically identical in each case.

Steady-state Operation Within the Phase of Logarithmic Growth

It was predicted earlier that it is difficult to maintain a continuous fermentation at a steady state within the phase of logarithmic growth because of the quasi-stable nature of the equilibrium in that region. However, in run 5 there were only small changes in bacterial density during 8 h of continuous operation in this region. The flow was held at $1/\theta = 0{\cdot}455$ h^{-1}, while the value of k_c predicted from the batch data was 0·455 for this pH of 5·4.

Nevertheless, the difficulties of operating at a steady state in this range are well illustrated in another run. During the first 7 h of continuous flow, the operation of a continuous fermentation at pH 6·0 (run 6) was allowed to be transient at $1/\theta = 0{\cdot}425$ h^{-1}. By equation (14), k_c during this period was found to be 0·48 h^{-1}, the same value as determined from earlier batch experiment.[16] With increased flow, a steady state was established at about $N = 2{\cdot}9$ U.O.D./ml with $1/\theta$ equal to 0·46 h^{-1}, slightly below the anticipated 0·48 h^{-1}. However, the system slowly drifted from this steady-state level and did not establish a new steady state during the period of operation. Small changes in the flow rate, as probably occurred here, are expected to cause the system to continue to drift from the original position for long periods rather than settling quickly at a new position. Steady-state operation was also achieved for short periods in the logarithmic growth region in other runs.

Transient Operation in the Logarithmic Growth Phase

Equation (14) was used to predict from batch data the bacterial densities after a period of continuous operation in the logarithmic phase not at steady state. The predicted values are compared to the observed experimental values in Table I, and tend to confirm the theoretical equations.

Table I. Experimental verification of equation (14) for predicting transient bacterial densities in continuous fermentation within the logarithmic growth phase

Run No.	N_i, U.O.D./ml	k, h^{-1}	$1/\theta$, h^{-1}	t, h	N, U.O.D./ml	
					predicted by eqn. (14)	Measured
6	1·68	0·48	0·43	7·00	2·5	2·5
7	0·94	0·48	0·18	3·00	2·3	2·2
8	1·97	0·48	0·40	4·00	2·7	3·0

Equation (14) can be modified to a convenient form for computing the logarithmic growth constant k_c, when using data obtained in continuous operation under transient conditions:

$$k_c = \frac{1}{\theta} - \frac{\ln N - \ln N_i}{t} \tag{14a}$$

Thus, one can determine kinetic constants from continuous fermentations, either steady state or transient. Such techniques may be particularly advantageous when the logarithmic phase is short and conditions change too rapidly in the batch fermentation for kinetic measurements.

Transient operation with $1/\theta$ set so that bacterial density increases very slowly has interesting possibilities, for it is, in effect, a 'fermentation in slow motion', where the effective specific growth rate is always $(k - 1/\theta)$.

Steady-state Conditions as Predicted by Graphical Methods

Continuous flow experiments confirm the validity of the graphical methods herein proposed for predicting continuous fermentation performance from batch data. For example, at pH 6·0 for a nominal holding time of 2·5 h, the experimental (run 9) and predicted (Fig. 1) values of steady-state bacterial density are respectively 5·5 and 5·35 U.O.D./ml.

With $1/\theta$ at 0·31 h^{-1} and a pH of 5·2 (run 3), a steady state was

reached at a bacterial density of 2·4 U.O.D./ml while the graphical method predicts a bacterial density of about 2·3 U.O.D./ml for these conditions. In operation without pH control (run 3), the first steady state attained was at pH 5·1 with $1/\theta$ at 0·36 h^{-1}. A pH of slightly less than 5·2 was predicted from batch data.[16]

A very interesting case of continuous fermentation was attempted where nearly all glucose entering in the feed was consumed and the level of glucose in the effluent was the same as that usually found after completion of batch fermentation. Presumably, the material that is analysed as glucose in effluent and final fermentation broths is a non-fermentable fraction which behaves as a reducing sugar in the analysis. It was found that the predicted and experimental results (run 7) differed somewhat, possibly because, as mentioned, the substrate was nearly exhausted and/or the cell death rate was becoming important. In this run at pH 6·0, a steady-state bacterial density of about 8·0 U.O.D./ml was maintained using $1/\theta = 0{\cdot}175$ h^{-1} in contrast to the predicted 0·14 for $1/\theta$. Conversely at $1/\theta = 0{\cdot}175$ h^{-1}, a bacterial density of 7·15 is predicted as compared to the measured 8·0. If the difference between the two levels is due to dead or inactive cells, the latter constitute over 10 per cent of the total population.

After continuous flow was stopped in the above experiment less than 1 ml of alkali was needed to neutralize the additional lactic acid formed batchwise in 1800 ml of medium and the bacterial density rose only 0·04 U.O.D./ml, an increase of less than $\frac{1}{2}$ per cent. This is further evidence that the medium was being completely fermented during the continuous operation.

The reducing sugar analysis of the feed stream showed 47·1 mg glucose/ml present. In the fermentor this was diluted by the alkali to an entering concentration of $47{\cdot}1 \times 264/321 = 38{\cdot}8$ mg/ml. In this run where about 0·8 mg of reducing sugar/ml were apparently unfermentable, $38{\cdot}8 - 0{\cdot}8 = 38{\cdot}0$ mg/ml were available for fermentation. By analysis, the lactic acid concentration was actually between 37·5 and 37·7 mg/ml, thus accounting for essentially all the available glucose and demonstrating that almost 100 per cent of the available glucose is converted to lactic acid in this fermentation.

Since equations (12) and (6) may be used outside the logarithmic

growth region for steady-state conditions, where k remains constant, the bacterial density can also be predicted as follows:

$$\frac{P}{N} = 2{\cdot}2 + 0{\cdot}53/0{\cdot}175 = 5{\cdot}22 \text{ mg lactic per U.O.D.}$$

$$N = 37{\cdot}6/5{\cdot}22 = 7{\cdot}2 \text{ U.O.D./ml.}$$

This prediction of 7·2 U.O.D./ml compares closely with the 7·15 predicted independently by the graphical method, both being somewhat below the experimental bacterial density of 8·0 to 8·1 U.O.D./ml. The difference may well be caused by the presence of dead or inactive cells not producing lactic acid at the normal rate.

Glucose and Lactic Acid Concentration During Continuous Operation

From equations (12) and (13) the concentrations of substrate and product in the effluent may be computed knowing the concentration of substrate in the feed and the bacterial density. These two equations are applicable to batch operation and continuous operation within the logarithmic phase, whether transient or steady-state conditions prevail, and also to steady-state conditions outside of the logarithmic range, where k also remains constant.

In Table II the concentrations of lactic acid and glucose as measured by analysis are compared to theory. An average value of 0·93 is used for p, the conversion efficiency. The agreement between experimental and predicted concentrations is very good (Table II), with the exception of a few cases where the lactic acid concentrations are low. These latter cannot be expected to be accurate since glucose is known to interfere with lactic acid analysis.[25] Hence, these data also confirm the equations and methods proposed.

Continuous Operation Without pH Control

Control of the pH was omitted in one experiment (run 3) in order to determine whether a continuous lactic acid fermentation would produce the steady cycling of population and pH as Finn

and Wilson[6] observed for other fermentations. No such cycling, however, was observed during the run; at two different flow rates the fermentation drifted slowly until it reached steady-state pH and bacterial density.

Continuous Operation Within the Lag Phase Region

Continuous fermentation at the very low bacterial densities which are usual in the lag phase of batch fermentations may or may not be of industrial interest, but it can be useful to the bacteriologist who wishes to measure growth rates at low populations where the lag phase might interfere.

Ordinarily, the lag phase is regarded as a period of adjustment,[12] during which the organisms endeavour to reach their maximum productivity by increasing the concentrations of intermediates and enzymes to optimal levels and perhaps counteracting toxic products transferred in the inoculum. The fermentation then enters its logarithmic phase where specific growth rate k, remains constant at its maximal value.

Since the lag phase is a temporary period of adjustment where growth rate is determined by factors other than the level of bacterial density, the batch data cannot be expected to predict continuous performance.

In a single exploratory experiment (run 10) high flow rates for the feed were used to force the fermentation back into that region corresponding to the lag phase of a batch fermentation. During the first five hours with $1/\theta = 0{\cdot}54$ h^{-1}, the bacterial density decreased from 0·88 to 0·68 U.O.D./ml. If k is assumed constant during this period, its value may be estimated as 0·49 h^{-1} from equation (14).

With a higher flow rate such that $1/\theta$ was 0·58 h^{-1}, N decreased further from 0·68 to 0·45. Again by calculation from equation (14), k was found equal to 0·485 h^{-1}.

The highest value for k measured in the corresponding batch fermentation was 0·455 h^{-1}, which is somewhat below the values computed above from continuous operation. Furthermore, at these low bacterial densities in the batch fermentation, k measured between 0·30 and 0·45 h^{-1}. Thus the experiment may be an indication that high specific growth rates would ordinarily

Table II. Comparisons of experimental and predicted concentrations of lactic acid and glucose during continuous fermentation

Run No.	Time, hours after inoculation	pH	k, h^{-1}	γ, mg/U.O.D.	mg/ml glucose in feed-stream	Flow Ratio, $\frac{\text{medium}}{\text{total}}$	S, mg/ml	N, U.O.D./ml	P, mg lactic/ml predicted[a]	P, mg lactic/ml analysis	S, mg glucose/ml predicted[b]	S, mg glucose/ml analysis
Steady-state Operation Within the Logarithmic Phase												
5	6·90	5·4	0·455	2·9	47·1	796/804	46·8	1·14	3·3	3·9	43·3	51·5
5	10·20	5·4	0·455	2·9	47·1	796/804	46·8	1·10	3·2	3·8	43·3	45·6
5	12·20	5·4	0·455	2·9	47·1	796/804	46·8	1·14	3·3	3·5	43·3	43·6
6	16·90	6·0	0·48	3·3	47·8	783/819	45·6	2·90	9·6	9·9	35·3	35·6
6	18·90	6·0	0·48	3·3	47·8	798/833	45·6	2·85	9·4	10·2	35·5	34·7
Steady-state Operation Beyond the Logarithmic Phase												
3	33·0	5·2	0·31	3·2	48·2	1·00	48·2	2·45	7·8	6·5	39·8	37·8
9	16·25	6·0	0·395	3·5	47·4	648/715	42·9	5·5	19·0	17·3	22·5	27·0
5	20·00	5·4	0·38	3·0	47·1	612/657	45·2	2·85	8·5	8·8	36·1	36·4

[a] By equation (12)

[b] By equation (13), $p = 0{\cdot}93$

Table II. Comparisons of experimental and predicted concentrations of lactic acid and glucose during continuous fermentation—(cont.)

Run No.	Time, hours after inoculation	pH	k, h^{-1}	γ, mg/U.O.D.	mg/ml glucose in feed-stream	Flow Ratio, medium / total	S, mg/ml	N, U.O.D./ml	P, mg lactic/ml predicted[a]	P, mg lactic/ml analysis	S, mg glucose/ml predicted[b]	S, mg glucose/ml analysis
						Transient Operation in the Logarithmic Phase						
1	7·40	5·6	0·45	3·3	48·2	882/897	47·4	1·70			41·3	39·4
1	7·75	5·6	0·45	3·3	48·2	882/897	47·4	1·70	5·6	5·6		
1	8·75	5·6	0·45	3·3	48·2	816/830	47·4	1·55	5·1	5·5	41·9	40·8
1	10·67	5·6	0·45	3·3	48·2	816/830	47·4	1·32	4·4	5·0	42·6	42·4
1	11·83	5·6	0·45	3·3	48·2	702/714	47·5	1·20	4·0	4·8	43·2	42·8
4	8·25	4·8	0·27	3·5	48·6	421/431	47·5	0·74	2·6	3·6	44·6	44·1
4	14·50	4·8	0·27	3·5	48·6	421/431	47·5	0·83	2·9	4·0	44·4	44·4
4	17·90	4·8	0·27	3·5	48·6	295/302	47·5	1·03	3·6	4·6	42·6	42·6
6	8·4	6·0	0·48	3·3	47·8	744/766	46·4	1·76	5·8	7·1	40·2	
6	12·1	6·0	0·48	3·3	47·8	744/766	46·4	2·30	7·6	8·1	38·2	38·3
6	14·2	6·0	0·48	3·3	47·8	744/766	45·9	2·60	8·6	10·0	36·7	37·3
6	21·6	6·0	0·48	3·3	47·8	798/833	45·6	2·27	7·5	6·1	37·5	35·0
7	6·0	6·0	0·48	3·3	47·6	308/327	45·0	0·94	3·1	4·6	41·7	42·5
7	9·1	6·0	0·48	3·3	47·6	308/341	43·0	2·25	7·4	8·3	35·0	36·5
7	12·1	6·0	0·48	3·3	47·6	308/357	41·2	3·84	12·7	15·0	27·5	29·5
9	6·00	6·0	0·48	3·3	47·4	700/726	45·5	1·70	5·7	6·1	39·4	39·2

[a] By equation (12)
[b] By equation (13), $p = 0·93$

prevail at the low bacterial densities in batch fermentation, but that these are nullified by lag phase effects. The above experiment suggests how continuous culture techniques may be useful in studying basic problems of bacteriology.

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Nomenclature

C, C'	Constants of integration
e	Base of natural logarithm, 2·71828 . . .
F	Volumetric flow rate, ml/h
k	Specific growth rate, h^{-1}
k_c	Specific growth rate in logarithmic phase (or logarithmic growth constant), h^{-1}
k_m	Maximum specific growth rate, h^{-1}
N	Bacterial density, units of optical density (U.O.D.) per ml
n	Number of continuous fermentation vessels in series
P	Concentration of fermentation product, mg/ml
p	Mass ratio of product formed to substrate consumed
q	A constant of proportionality for the effect of toxic products on specific growth rate.
S	Substrate concentration, mg/ml
t	Time, h
V	Liquid volume in continuous fermentor, ml
α, β, γ	Constants
θ	Nominal holding time, h

Subscripts

0	Refers to conditions in the feed stream
$1, 2, 3, \ldots, n$	refers to conditions within the 1st, 2nd, 3rd, . . ., nth fermentors respectively, or to their effluents

G	Refers to changes due to bacterial growth
i	Refers to initial conditions when time is zero
s	Refers to steady-state conditions
t	Refers to toxic products.

References

1 Adams, S. L. and Hungate, R. E. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 1815
2 Bilous, O. and Piret, E. L. *A.I.Ch.E. Journal*, 1 (1955), 480
3 Eldridge, J. W. and Piret, E. L. *Chem. Engng. Progr.*, 46 (1950), 290
4 Elsworth, R. and Meakin, L. R. P. *Chem. & Ind.* (*Rev.*) (1954), 926
5 Ericcson, E. O. *Chem. Engng. Progr.*, 43 (1947), 165
6 Finn, R. K. and Wilson, R. E. *J. Agr. Food Chem.*, 1 (1953), 66
7 Friedemann, T. E. and Graeser, J. B. *J. biol. Chem.*, 145 (1932), 131
8 Gaden, E. L., Jr. *Chem. Engng. Progr.*, 51 (1955), 540
9 Gaden, E. L., Jr. *Chem. Engng.*, 63, No. 4 (1956), 159
10 Golle, H. A. *J. Agr. Food Chem.*, 1 (1953), 789
11 Hildago Fernandez-Cano, L. and Reus Cid, A. *Rev. Cienc. apl.*, 6 (1952), 37, 124
12 Hinshelwood, C. N. *The Chemical Kinetics of the Bacterial Cell.* (1946). London; Oxford University Press
13 Hodge, J. E. and Davis, H. A., U.S. Dept. of Agriculture, Bur. *Agric. Industr*, Chem., AIC–333 (1952)
14 Jones, R. W. *Chem. Engng. Progr.*, 47 (1951), 46
15 Luedeking, R., Ph.D. Thesis, Dept. of Chemical Engineering, University of Minnesota (1956)
16 Luedeking, R., and Piret, E. L. This Journal, 1 (1959), 393
17 MacDonald, R. W. and Piret, E. L. *Chem. Engng. Progr.*, 47 (1951), 363
18 Mason, D. R. and Piret, E. L. *Industr. Engng. Chem.* (*Industr.*), 42 (1950), 817
19 Maxon, W. D. *Appl. Microbiol.*, 3 (1955), 110
20 Monod, J. *Annu. Rev. Microbiol.*, 3 (1949), 371
21 Monod, J. *Ann. Inst. Pasteur*, 79 (1950), 390
22 Nelson, N. *J. biol. Chem.*, 153 (1944), 375
23 Novick, A. and Szilard, L. *Proc. nat. Acad. Sci., Wash.*, 36 (1950), 708
24 Powell, E. O. *Chem. & Ind.* (*Rev.*), (1954), 928
25 Smith, R. J. *Analyt. Chem.*, 25 (1953), 505
26 Somogyi, M. *J. biol. Chem.*, 195 (1952), 19
27 Weber, A. P. *Chem. Engng. Progr.*, 49 (1953), 26